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14. ABSTRACT

Since tumor cells in general are genomically unstable and have defects in DNA damage responses, it has been proposed that targeting DNA repair pathways may lead to a therapeutic index in tumor cells over "normal" cells. Previous studies have demonstrated that BRCC36 is over-expressed in the vast majority of invasive breast cancers and that depletion of BRCC36 sensitizes breast cancer cells to IR via the BRCA1 DNA repair pathway. Therefore, we hypothesize that abrogation of BRCC36 will sensitize breast tumors to the DNA-damage based therapies. To test this hypothesis, we will utilize an antibody (anti-HER2)-protamine based siRNA delivery system to selectively deplete BRCC36 in breast tumor xenografts. This cancer cell-specific or "smart" therapeutic approach should improve the targeting of breast tumor cells while reducing non-specific toxicity. The proposed studies will clearly establish BRCC36 as a novel therapeutic target to enhance the efficacy of radiation and chemotherapy which elicit DNA damage. As the antibody is currently being developed for ImmunoPET imaging trials in breast cancer patients, clinical translation of successful preclinical results of our antibody-P/siRNA conjugates could be rapidly achieved.

15. SUBJECT TERMS

BRCA1, BRCC36, and siRNA target therapies

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INTRODUCTION

Although breast cancer-related death rates have decreased somewhat during the last decade, it is expected that breast cancer will be diagnosed in over 182,000 women in the United States in 2008 and it is estimated that 13.2 % of all American women (1 in 8) will develop breast cancer and 3.0 % will die from this disease (Jemal, et al., 2008; Ries, et al., 2008). Breast cancer is responsible for about 15% (>40,000) of all female cancer deaths in the same year, second only to lung cancer-related deaths in the US (Jemal, et al., 2008). With an estimated 1.2 million new cases diagnosed and nearly 410,000 breast-cancer related deaths per year, this disease is the single most common life-threatening cancer to affect women worldwide (Bray, et al., 2004; Parkin, et al., 2005).

The BRCA1 gene (OMIM: 113705) is one of the most intensively studied breast cancer susceptibility genes and has a profound role in breast cancer etiology owing to its involvement in several important cellular processes. Importantly, estimates from previous studies (Collaborative Group on Hormonal Factors in Breast Cancer, 2001; Margolin, et al., 2006) indicate that family history is associated with 15% to 20% of breast cancer cases in the United States. Therefore, most familial aggregation of breast cancer remains unexplained. Furthermore, recent findings of phenotypic overlap between *BRCA1*-associated and sporadic basal-like breast cancers suggest that the latter might have an underlying defect in BRCA1-related pathways (Foulkes, et al., 2003; Lakhani, et al., 2005; Sorlie, et al., 2003; Turner, et al., 2007). Therefore, dysfunction of other genes, which code for proteins in complementary pathways as BRCA1, could be important in the pathogenesis of a significant proportion of sporadic breast cancers.

BRCA1-Associated Proteins: Functional Modifiers of BRCA1

The *BRCA1* gene encodes for a 220 kDa nuclear phosphoprotein that has been suggested to play a role in maintaining genomic stability and to act as a tumor suppressor (Miki, et al., 1994). Findings from mouse studies demonstrated that *Brca1* knockout mice, generated by removal of exon 11, have a defective G_2/M cell cycle checkpoint and extensive chromosomal abnormalities, and developed mammary tumors (Xu, et al., 2001; Xu, et al., 1999). BRCA1 interacts directly or indirectly with other tumor suppressors (such as p53 and BRCA2), DNA damage sensors (such as RAD51, RAD50, MRE11 and NBS1), ubiquitin ligase partners (BARD1, BRCC45, BRCC36), and signal transducers (such as p21 and cyclin B) to form multi-subunit protein complexes, such as BASC (BRCA1-associated genome surveillance complex) and BRCC [**Figure 1,** (Chen, et al., 2006a)]. These multi-subunit protein complexes are involved in a broad range of biological processes including DNA repair, cell cycle control, ubiquitination, and chromatin remodeling (Chen, et al., 2006a). The majority of BRCA1 functional studies have focused on its potential role in DNA

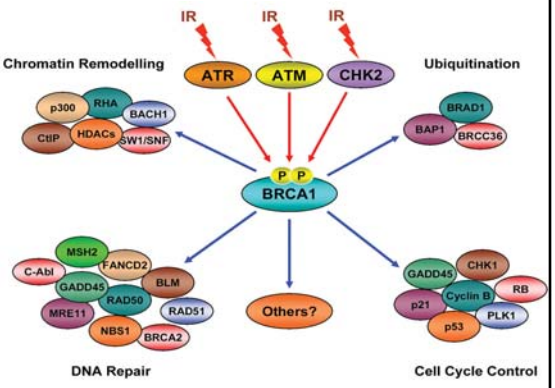

Figure 1. BRCA1-associated protein network. BRCA1 interacts with a number of proteins to form multi-subunit complexes. BRCA1-associated protein complexes are involved in DNA repair, protein ubiquitination, cell-cycle-checkpoint control, and chromatin remodeling (Chen et al, 2006a).

damage responses. The implication that BRCA1 is a direct component of DNA damage response pathways comes from evidence of its interactions with BRCA2 and RAD51. The protein complex comprised of BRCA1, BRCA2, and RAD51 has been shown to activate DNA double-strand break (DSB) repair and to initiate homologous recombination, an observation which links the maintenance of genomic integrity to tumor suppression (Chen, et al., 1999). In addition, the BRCA1-associated MRE11-RAD50-NBS1 (MRN) complex has recently been demonstrated to activate CHEK2 downstream from ATM in response to replication-mediated DSBs (Takemura, et al., 2006). Disruption of any of these pathways may contribute to increased genomic instability and potentially sensitize cells to the effects of ionizing radiation (IR), specifically through the induction of cellular apoptosis. BRCA1 also interacts with a number of proteins and displays significant ubiquitin ligase activities. Importantly, deleterious mutations affecting the BRCA1 RING-finger domain, which were found in clinical specimens, abolish the ubiquitin ligase activity of BRCA1 (Ruffner, et al., 2001; Wu, et al., 1996). These findings support a relationship between the ligase activity of BRCA1 and the predisposition to breast cancer. In addition, BRCA1 has also been reported to interact with the RNA Pol II holoenzyme (Scully, et al., 1997). Two recent reports have suggested that BRCA1and BARD1 may be involved in the degradation of RNA polymerase II complex and siRNA-mediated knockdown of BRCA1 and BARD1 results in stabilization of RNAP II in the cells following UV exposure (Kleiman, et al., 2005; Starita, et al., 2005). These studies reported that BRCA1/BARD1 appears to initiate the degradation of stalled RNAP II and thus disrupts the coupled transcription by inhibiting RNA processing machinery in cells exposed to DNA damage.

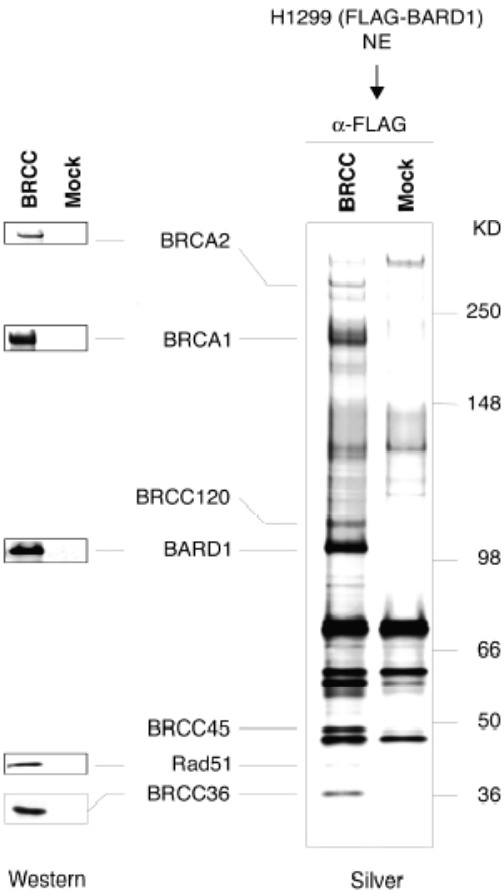

Figure 2. Purification of BRCC. Analysis of anti-FLAG eluate by SDS-PAGE followed by silver staining (Dong, 2003).

BRCA1-Associated Proteins: Potential Targets of Breast Cancer Therapies

Considerable efforts have been made toward understanding the mechanism of the response to both cytotoxic chemotherapy and radiation therapy in the treatment of breast cancer. Tumor cells in general are genomically unstable and have defects in DNA damage responses. It has been proposed that targeting DNA repair pathways may lead to a therapeutic index in tumor cells over "normal" cells. Because of the important role of BRCA1 in DNA repair, breast tumor cells with defective BRCA1 are believed to be more sensitive to DNA-damage based therapies (Farmer, et al., 2005). This speculation is supported by the recent development of the inhibitors of poly (ADP-

ribose)-polymerase-1 (PARP). The PARP enzyme is involved in base excision repair which is a critical pathway in the repair of DNA single-strand breaks (Ratnam and Low, 2007; Schreiber, et al., 2002). Farmer and colleagues have shown that defects in BRCA1 or BRCA2 profoundly sensitize cells to the inhibition of PARP enzymatic activity, resulting in chromosomal instability, cell cycle arrest, and subsequent apoptosis (Farmer, et al., 2005). PARP inhibitors are currently in clinical trials of patients with breast cancer or other malignancies who are *BRCA1* or *BRCA2* mutation carriers. Two phase I studies have shown that AZD2281 (AstraZeneca, UK), a potent orally active PARP inhibitor, is well tolerated and leads to significant PARP inhibition in patients carrying *BRCA1* and *BRCA2* mutations with breast or ovarian cancer (Fong, et al., 2008; Yap, et al., 2007). Importantly, clinical responses have been observed in all cohorts evaluated thus far, and future phase II studies are planned (Fong, et al., 2008; Yap, et al., 2007). Findings from these recent studies further suggest that the design of novel therapies, which inhibit components of particular DNA repair pathways, may provide effective and more tolerable therapeutic options for breast cancer patients with BRCA1 defects.

Nevertheless, defects in BRCA1 itself may not be the only reason for the loss of its activity nor the increased sensitivity of tumor cells to DNA damage-based agents. A number of studies have demonstrated that manipulation of BRCA1-associated proteins, such as RAD51, MRE11, and NBS1, can impact cellular sensitivity to ionizing radiation (IR) (Chen, et al., In press; Digweed, et al., 2002; Lio, et al., 2004; Nakanishi, et al., 2002; Russell, et al., 2003). BRCA1-associated proteins may, therefore, be considered as potential targets for breast cancer therapies. Despite a potentially significant role for BRCA1-associated protein complexes in modifying the activities of BRCA1, the total number of complexes and the identity and function of component proteins has yet to be fully elucidated. Thus, much of the scientific effort related to BRCA1 is currently directed at defining the biochemical functions of BRCA1 in association with these protein complexes.

BRCA1/2 Containing Complex (BRCC)

Wang *et al* have previously reported that a set of proteins associate with BRCA1 to form a large mega-Dalton protein complex, referred to as BASC (BRCA1-Associated Genome Surveillance Complex) (Wang, et al., 2000). This complex includes several DNA damage repair proteins, MSH2, MSH6, MLH1, ATM, the MRE11-RAD50–NBS1 protein complex, and the RFC1-RFC2-RFC4 complex. BASC is responsive to double stranded breaks (Wang, et al., 2000). In addition, BRCA1 directly interacts with the brahma-related gene 1 (BRG1) subunit of SW1/SNF-associated complex which has been demonstrated to be involved in chromatin-remodeling (Bochar, et al., 2000). However, it becomes clear that BRCA1 and/or BRCA2 can exist in a number of protein complexes and that many BRCA1/2 associated proteins remain to be identified.

Using a combination of affinity purification of anti-FLAG and mass spectrometric sequencing, we have reported a novel multiprotein complex, termed BRCC (BRCA1/2 Containing Complex) (**Figure 2**), which contains seven polypeptides including BRCA1, BRCA2, BARD1 and RAD51 (Dong, et al., 2003). We first reported that BRCC was an E3 ubiquitin ligase complex exhibiting activities in the E2-dependent ubiquitination of the tumor suppressor p53. In this multiprotein complex, three proteins, referred to as BRCC36, BRCC45, and BRCC120 have been found to be associated with BRCA1 and BRCA2.

Mass spectrometric sequence analysis of the 36-kDa bands in **Figure 2** identified a protein referred to as BRCC36 (Dong, et al., 2003). *BRCC36/c6.1A* gene is located at the Xq28 locus, a chromosomal break point in patients with prolymphocytic T-cell leukemia (T-PLL) (Fisch, et al., 1993). The chromosomal break occurred in two different introns of *BCC36/c6.1A* and the fusion transcripts were expressed at high levels in the leukaemic cells from T-PLL patients (Fisch, et al., 1993). The *BRCC36/C6.1A* gene is highly conserved between species and bears sequence homology with both human Poh1/Pad1 subunit of the 26S proteasome and subunit 5 (Jab1) of the COP9 signalosome (Dong, et al., 2003). Despite its homology to POH1 and Jab1, BRCC36 represents a distinct branch in the evolutionary tree. We have demonstrated that depletion of BRCC36 resulted in increased sensitivity in breast cancer cells to IR and disruption of IR-induced BRCA1 phosphorylation and nuclear foci formation (Chen, et al., 2006b). RNA interference of BRCC36 also resulted in a defect in G2/M checkpoint arrest (Dong, et al., 2003). Cancer-associated truncations in BRCA1 have been found to reduce the association of BRCC36 with the BRCC complex (Dong, et al., 2003). In addition, our previous study has shown that a recombinant four-subunit BRCC complex containing BRCA1-BARD1-BRCC45-BRCC36 revealed an enhanced E3 ubiquitin ligase activity compared to that of BRCA1-BARD1 heterodimer (Dong, et al., 2003). Therefore, BRCC36 appears to be a positive regulator of BRCA1/BARD1 E3 ligase activity. Furthermore, BRCC36 has recently been reported to also be present in a novel protein complex, BRCA1-RAP80-ABRAXAS-BRCC36 (BRCA1 A complex), and displays deubiquitinating (DUB) activities (Sobhian, et al., 2007; Wang and Elledge, 2007). The recruitment of BRCC36 to this complex is via the interaction between the coiled-coil domains of BRCC36 and ABRAXAS. BRCC36 plays an important role in BRCA1 A complex, and it is essential for the localization of RAP80, ABRAXAS, and BRCA1 to sites of DNA damage. These findings suggest that the balance between synthesis and turnover of certain polyubiquiquitinated structure by BRCA1-BARD1 E3 and BRCC36 DUB activities, respectively, could be dynamic and mediated by other protein partners (e.g., BRCC45 or RAP80) in the same complexes.

Antibody-Mediated siRNA Delivery

Considerable research efforts have been focused on applying siRNA for human disease therapy, including cancer therapy. A novel method for *in vivo* delivery of siRNAs to specific cell types has been recently developed, and it takes advantages of the nucleic-acid binding properties of protamine as well as the specificity of fragment antibodies (Fab) (Sioud, 2006). This method shows that systemically administered siRNA can be targeted to cells that express a specific cell-surface receptor (Peer, et al., 2007; Song, et al., 2005). Compared to other siRNA delivery systems, antibody-based siRNA targeting provides many advantages (Sioud, 2006), including that (i) the siRNA is stable in the blood with a prolonged half-life; (ii) the siRNA can be transported across capillary endothelial walls; (iii) the siRNA can be specifically bound to the plasma membranes of target cells ("smart drug"); and (iv) the siRNAs can be efficiently delivered into the target cells through endocytosis. Here, we will apply a cancer cell-specific or "smart" therapeutic approach utilizing diabody-P/siRNA conjugates that should lead to an improvement in the targeting of breast tumor cells, while reducing non-specific toxicity.

BODY

Task 1: To Express and Purify Anti-HER2 C6.5-Protamine Fusion Protein.

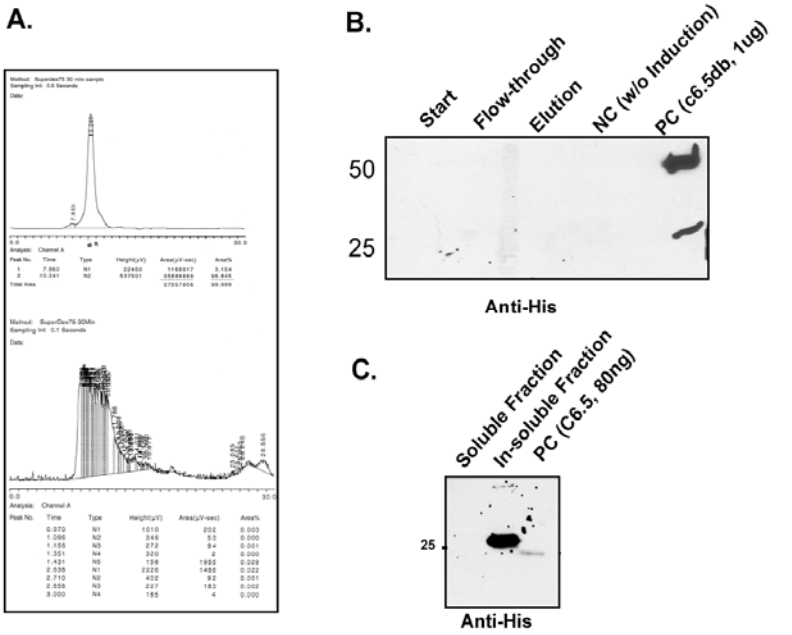

Figure 3. Purification of C6.5–protamine fusion proteins. Purification of C6.5-protamine fusion proteins was determined by HPLC (**A.** Up panel: C6.5; Low panel: C6.5-protamine) and Western blot analyses (**B**). (**C**) The levels of C6.5-protamine fusion protein were also examined by Western blot assay in protein samples extracted from soluble and insoluble cell fractions. Recombinant C6.5 protein was loaded as positive control (PC).

Expression of anti-HER2 C6.5db-P fusion protein:

The sequence of the truncated form of human protamine (amino acids 8-29: RSQSRSRYYRQRQRSRRRRRRS) was first subcloned between the C-terminus of the anti-HER2 C6.5 and His-tag in the vector pCYN2. Anti-HER2 C6.5-P-His was then expressed in TG1 E. coli, extracted in periplasmic extraction buffer (30 mM Tris-HCl, 20% sucrose, 1mM EDTA). After centrifugation, anti-HER2 C6.5-P-His fusion protein in the soluble fraction was purified using the Ni-nitrilotriacetic acid (Ni-NTA) agarose followed by high-performance liquid chromatography (HPLC) size-exclusion chromatography over a Superdex 75 column (Amersham Pharmacia). HPLC and Western blot analyses showed that no trace of C6.5-P-His fusion protein was found in the flowthrough and elusion solution (**Figure 3A and 3B)**. Further Western analysis showed that the abundant C6.5-protamine-His was detected in the insoluble fraction of extraction buffer. However, the C6.5-protamine-His fusion protein was not detected in the soluble fraction at all (**Figure 3C**). These results indicated that the C6.5-Protamine-His fusion protein was insoluble in current extraction buffer. As shown in **Figure 4A** and **4B**, only strong denaturants such as, 6 M GuaHCl, 2% SDS, and 1% Triton X-100, were able to efficiently extract the fusion proteins C6.5-protamine. As 2% SDS and 1% TRitonX-100 may cause irreversible denaturation of the proteins, we chose 6 M GuaHCl to extract fusion proteins from insoluble section as described in a previous study (Li, et al., 2001). However, the yield of purified fusion protein was still too low to perform the siRNA delivery experiments (data not show). Based on the facts that protamine has strong binding capacity with the nucleotides, we have found that protamine fragment (a.a., 8-29) is a high charge molecular. As shown in Figure

3, the C6.5-protamine fusion protein has a charge value of +15.9 at pH7.0 in comparison to that C6.5 alone only has a positive charge of 3.7 at pH 7.0. This high charge character of protamine fragment (a.a., 8-29) appears to be responsible for the insolubility of C6.5-protamine fusion protein.

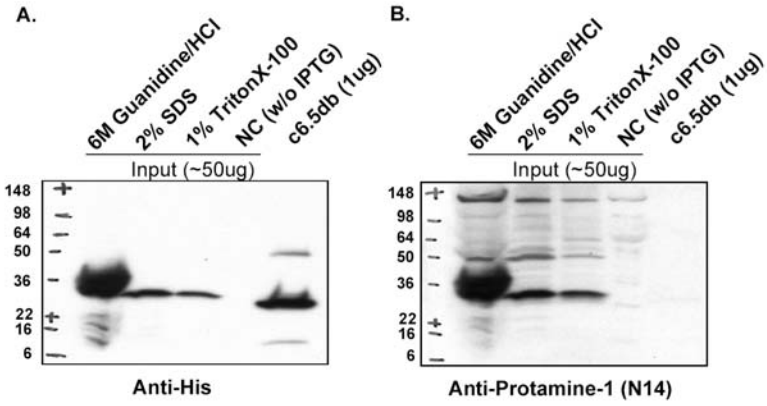

Figure 4. Detection of C6.5–protamine fusion proteins. Protein samples were first extracted from bacterial lysed with various reagents including 6 M GuaHCl, 2%SDS, or 1% Triton X- 100. The lysate from non-induced bacterial was used as negative control (NC), and recombinant C6.5 protein was loaded as positive control (PC). Protein samples were then fractionated in SDS-PAGE followed by Western blot analysis using antibodies against His tag (**A**) and protamine (**B**).

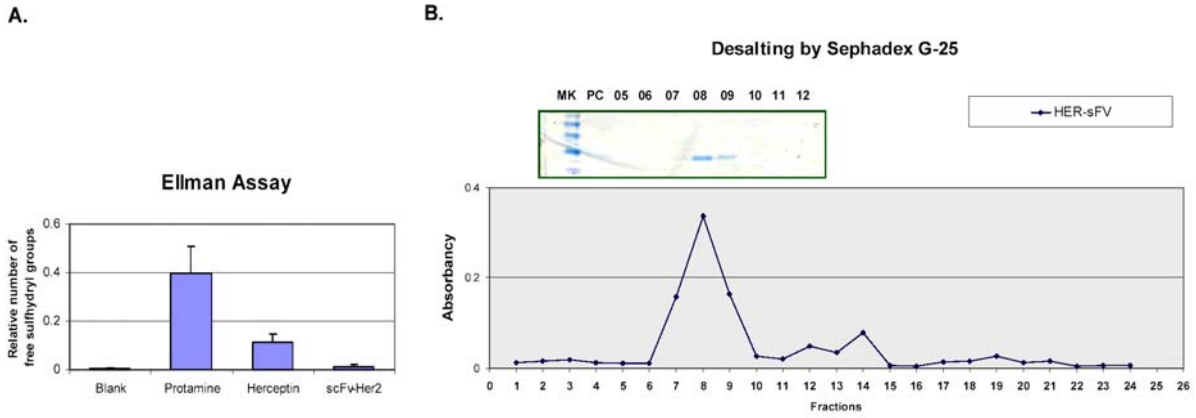

Figure 5. Reduction of anti-HER2 scFv3.9. (**A**) Ellman's assay for the determination of free sulfhydryl groups. (**B**) Fractionation in desalting by Sephadex G-25.

Conjugation of anti-Her2 antibody and protamine peptide

Due to the technical difficulties of purifying the anti-HER2 C6.5–protamine fusion protein, we have decided to use an alternative approach to first synthesize the protamine (a.a., 8-29)-Cys and then conjugate the protamine-Cys with cysteine-containing anti-HER2 antibodies. Because of the character of high charge in protamine peptide (a.a., 8-29), the scientists in Genscript took 3x tries to finally synthesize this peptide. We have first measured the baseline levels of free -SH group containing in cysteine using 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent; Pierce). As shown in **Figure 5A**, the -SH contents is much higher in the protamine and Herceptin, an anti HER2 antibody, but pretty low in anti-HER2-scFV3.9, which contains two cysteines in its sequence. Therefore, the protein of anti-HER2 scFv3.9 was first reduced by DTT to obtain

monomer anti-HER2 sFv3.9-SH. In brief, 1M DTT was added to the scFv in buffer of 10 mM HEPES and 150 mM NaCl, (pH 7.4) to a final concentration of 50 mM. After rotation at room temperature for 5–10 min, the protein was desalted on a Sephadex G-25 column (GE) (**Figure 5B**). To perform the conjugation, protamine (a.a., 8-29)-Cys was incubate with Herceptin or scFv3.9 at molecular ration of 10:1, respectively. The solution was then mixed by gentle rotation for 30 min at room temperature to produce HER2 antibody and Protamine conjugation.

Task 2: To Determine if Abrogation of BRCC36 by C6.5-P siRNA Delivery can Sensitize Breast Tumors to DNA Damage-Based Therapies in Mouse Xenograft Models.

BRCC36 siRNA delivery in HER2-positive cells via anti-HER2 antibodies and protamine conjugates

To examine if HER2 antibodies and protamine conjugates enable delivery of *BRCC36* siRNA specifically to the HER2-positive breast cancer cells, we have performed *in vitro* silencing studies in the SKBR-3 breast cancer cell line which constitutively expresses high levels of HER2 and BRCC36 (Chen, et al., 2006b). In comparison to control cells without siRNA treatment, Western blot analysis revealed a ~50% decrease in BRCC36 levels in the cells which siRNAs were delivered by either lipid (i.e., Oligofectamine, Invitrogen) or the conjugates of Herceptin and protamine peptide transfected cells (**Figure 6**). However, the breast cancer cells added with the conjugates of anti-HER2 scFv3.9 and protamine peptide have no response to the BRCC36 siRNA treatment. Because of the low levels of free -SH group presented in anti-HER2 scFV3.9, we expect the conjugation between anti-HER2 scFv3.9 and protamine may be less effective. We are currently optimizing the condition for reduction of scFv in order to increase the level of free –SH in anti-HER2 scFv protein.

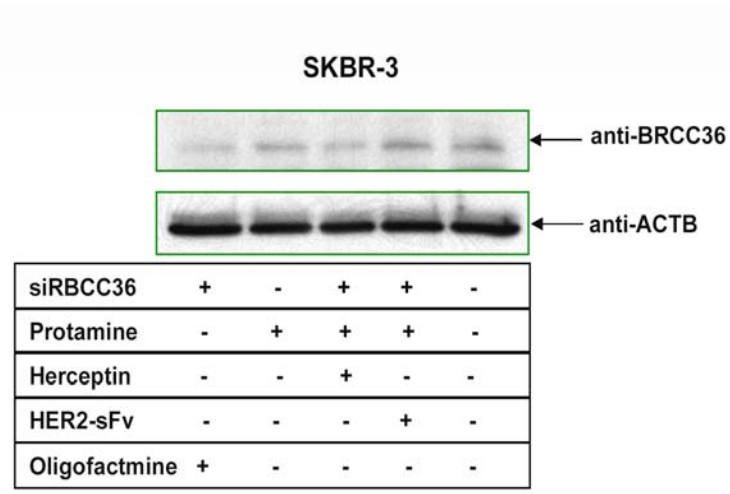

Figure 6. Knocking-down BRCC36 expression by siRNA delivery via anti-HER2 antibody-protamine conjugates. For the siRNA delivery studies, SKBR-3 cells were plated at a density $5x10^3$ cells/cm^2. After reaching 30% to 40% confluence, cells were transfected with *BRCC36* siRNA using either oligofectamine or antibody/protamine conjugates in OPTI reduced serum medium. After 48 hours, cells were harvested, and Western blot analysis was performed to examine the protein level of BRCC36 in cell lysates. Protein loading level was evaluated by immunoblotting with anti-β-actin antibody.

Exogenous BRCC36 expression stimulates colony formation and sensitizes breast cells to IR-induced apoptosis

We next evaluated whether exogenous expression of BRCC36 alone could transform MCF-10F, a non-tumorigenic mammary epithelial cell line, initially by assessing anchorage-independent growth. As shown in **Figure 7**, FLAG-tagged BRCC36-overexpressing MCF-10F cells formed more colonies (>30 cells after 3 weeks) in soft agar, as compared to vector-control MCF-10F cells. The breast tumor cell line, MCF7 was included as a positive control. After quantification, BRCC36-overexpressing MCF-10F cells are ~5-times more efficient in colony formation than vector-control cells (18.5 ± 2.1 vs. 3.5 ± 0.7, $p < 0.01$) (**Figure 7**). In addition, we have hypothesized that the level and/or integrity of BRCC36 could be a critical factor that mediates BRCA1-associated DNA damage response. We have previously shown that disruption of BRCC36 via siRNA-depletion blocks BRCA1 activation and enhance IR-induced apoptosis in breast cancer cells (Chen, et al., 2006b). We recently evaluated whether aberrant expression (i.e., over-expression) of BRCC36 in non-tumorigenic breast cells would induce a similar phenotype as depletion of BRCC36 in response to IR-induced apoptosis. Following transient transfection of a *BRCC36* expression construct (**Figure 8A**), MCF-10A cells were exposed to 4-Gy of total IR

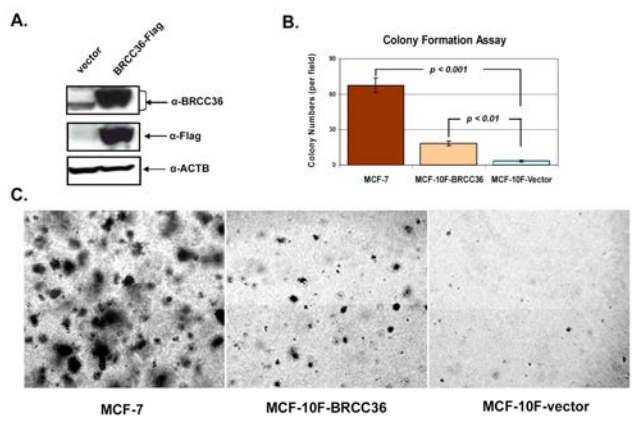

Figure 7. Exogenous overexpression of BRCC36 stimulates colony formation in MCF-10F cells. (**A**) The expression of BRCC36 in MCF-10F cells transfected with either a control vector (pFLAG-CMV2-5a) or a BRCC36-expression vector (pFLAG-CMV2-BRCC36) was determined by immunoblotting with either anti-BRC36 or -FLAG antibodies. (**B**) 5 x 10^5 of MCF-7 (positive control), BRCC36 transfected- or vector control MCF-10 cells were plated in soft agar (6-well plates). After 3 weeks, images from 5 independent fields of each well were taken and colonies containing >30 cells were scored. The numbers of colonies shown are the means ± standard deviations (SD) of triplicate results from two independent experiments. (**C**) Representative images of colony formation in soft agar by MCF-7, BRCC36-transfected and vector control MCF-10F cells.

utilizing a Cesium 137 Irradiator (Model 81-14R). Cells were cultured for an additional 72 hours prior to harvesting and were examined for DNA damage-induced cell apoptosis via Annexin V and 7-amino actinomycin D staining. The proportion of apoptotic cells was determined utilizing a Guava Personal Cytometer (Guava Technologies) as we have described previously (Chen, 2006). As expected, no significant difference in the fraction of cells undergoing apoptosis in vector- or *BRCC36*-transfected cells was observed in the absence of IR (4.6% ± 0.2% vs. 4.8% ± 0.6%) (**Figure 8B, upper panels**), i.e., enhanced expression of BRCC36 alone is not in itself toxic. However, when combined with IR exposure, these cells show a significant increase in the fraction of apoptotic cells (44.2% ± 15.4%) when compared to the vector control group (17.8% ± 3.4%, $p<0.05$) (**Figure 8B, lower panels**). Consistent with these results, the overall cell viability was substantially lower in *BRCC36*-overexpressed cells following IR as compared to

vector control cells (52.1% ± 14.6% vs. 81.9% ± 3.5%, *p<0.05*) (**Table 1**). As we have reported previously, disruption of BRCC36 leads to enhanced IR-induced cell death. These data suggest that aberrant expression of BRCC36 can also disrupts BRCA1-associated DNA repair pathways and sensitizes cells to IR-induced apoptosis. These findings support our hypothesis that the aberrant expression (gain or loss) or activity of protein(s) in BRCA1-associated pathways will lead to a BRCA1 null-like phenotype and DNA damage hypersensitivity in breast cancer cells.

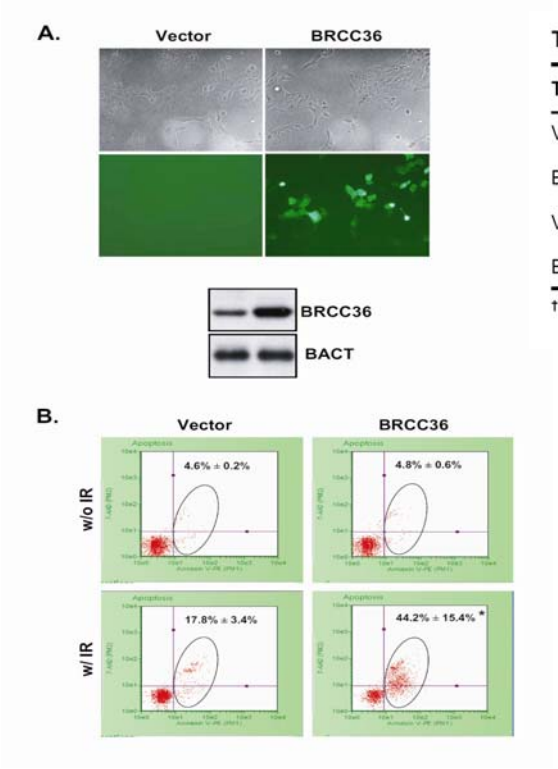

Table 1. Nexin Assay in MCF-10A Cells

Transfection	IR	Viable Cells	Apoptotic Cells
Vector	w/o	95.0% ± 0.2%	4.6% ± 0.2%
BRCC36	w/o	94.5% ± 0.4%	4.8% ± 0.6%
Vector	w/	81.9% ± 3.5%	17.8% ± 3.4%
BRCC36	w/	52.1% ± 14.6%†	44.2% ± 15.4%†

†: BRCC36 transfection vs. vector control (Student's *t*-test, $p<0.05$)

Figure 8. Exogenous overexpression of BRCC36 sensitizes MCF-10A cells to IR-induced apoptosis. (**A**) The expression of BRCC36 in MCF-10A cells transfected with either a control vector (pRetroX-zsGreen1) or a BRCC36-expression vector (pRetroX-zsGreen1-BRCC36) was determined by GFP expression and immunoblotting with anti-BRCC36. Protein loading levels were evaluated by immunoblotting with anti-β-actin antibody (BACT). (**B**) The proportion of apoptotic cells was measured following annexin V and 7-amino actinomycin D staining using a Guava Personal Cytometer. All studies were done in triplicate.

KEY RESEARCH ACCOMPLISHMENTS

- Subcloned the fragment of Protamine (a.a., 8-29) between the C-terminus of the anti-HER2 C6.5 and His-tag in the vector pCYN2.

- Expressed the anti-HER2 antibody (C6.5)- protamine fusion protein in TG1 E. coli.

- Demonstrated that anti-HER2 antibody (C6.5) -protamine fusion protein is a molecular which has very high positive charge and is not soluble in non-denature buffer.

- Synthesized protamine (a.a., 8-29) with additional cysteine, and performed conjugation between anti-Her2 antibodies and protamine peptide

- Demonstrated that siRNA delivered via the conjugates of Herceptin and protamine peptide enable knock-down of the level of BRCC36 in the HER2-positive breast cancer cells.

- Demonstrated that exogenous BRCC36 expression stimulates colony formation and sensitizes breast cells to IR-induced apoptosis.

REPORTABLE OUTCOMES

Abstracts

1. **Chen, X**, Weaver, J, Bove, BA, Vanderveer, L, Miron, A, Daly, MB, Godwin. AK. Allelic imbalance in BRCA1 and BRCA2 gene expression is associated with an increased breast cancer risk. In: Era of Hope, Department of Defense (DoD) Breast Cancer Research Program Meeting, (AB# 32-11, poster presentation), 2008.

2. **Chen, X**, Amin, N, Godwin. AK. Abrogation of BRCC36 impairs IR-induced BRCA1 activation and sensitizes breast cancer cells to IR-induced apoptosis. In: Era of Hope, Department of Defense (DoD) Breast Cancer Research Program Meeting, (AB# 32-7, poster presentation), 2008.

3. **Chen, X**, Weaver, J, Bove, BA, Vanderveer, L, Miron, A, Daly, MB, Godwin, AK. Allelic imbalance in BRCA1 and BRCA2 gene expression is associated with an increased breast cancer risk. In: Annual meeting of American Association of Cancer Research, (AB# 1926, poster presentation), 2008.

4. **Chen, X.**, Klimowicz, C., Vanderveer, L., Weaver, J., Amin, N., Ouellette, T., Liao, C., Daly, M.B., Nathanson, K.L., Godwin, A.K. A BRCA1 5'non-coding variant influences breast cancer risk among African-Americans; In: Annual meeting of American Association of Cancer Research; (AB# 4239, poster presentation), 2009.

Publications

1. **Chen, X**, Weaver, J, Bove, BA, Vanderveer, LA, Weil, SC, Miron, A, Daly, MB, Godwin, AK. Allelic Imbalance in BRCA1 and BRCA2 Gene Expression Is Associated with an Increased Breast Cancer Risk. Hum Mol Genet, 17: 1336-1348, 2008.

2. **Chen X,** Kistler JL, Godwin AK. BRCA1-associated proteins: novel targets for breast cancer radiation therapy. In: Columbus F., editor. Radiation therapy for breast cancer. Hauppauge, NY: Nova Science Publishers, Inc., in press.

CONCLUSIONS

In cellular response to the DNA damage caused by IR, ATM is activated by DNA damage and phosphorylates multiple factors, including BRCA1 and p53, which are involved in DNA repair, apoptosis and cell cycle arrest. As our results indicate, depletion of BRCC36 expression by

RNAi blocks BRCA1 activation (i.e., phosphorylation and nuclear foci formation) in breast cancer cells following IR exposure. Because of the role of BRCA1 in DNA repair, we propose that an imbalance between the DNA repair/cell survival and DNA damage/cell apoptosis pathways exists in BRCC36-depleted cells following IR exposure. As a result, BRCC36 depletion appears to substantially sensitize breast cancer cells to IR-induced apoptosis. Therefore, we are examining if abrogation of BRCC36 will sensitize breast tumors to the DNA-damage based therapies. We have tested a cancer cell-specific or "smart" therapeutic approach utilizing the conjugation of anti-HER2 antibodies and protamine to deliver BRCC36 siRNA to HER2 positive breast cancer cells. Since tumor cells in general are genomically unstable and have defects in DNA damage responses, it has been proposed that targeting DNA repair pathways may lead to a therapeutic index in tumor cells over "normal" cells. This approach should lead to improving the targeting of breast tumor cells while reducing non-specific toxicity.

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Yap TA, Boss DS, Fong PC, Roe lvink M, T utt A, Carm ichael J, O'Connor MJ, Kaye SB, Schellens JH, De Bono JS. 2007. First in hum an phase I pharm acokinetic (PK) and

pharmacodynamic (PD) study of KU-0059436 (Ku), a small molecule inhibitor of poly ADP-ribose polymerase (PARP) in cancer patients (p), including BRCA1/2 mutation carriers. J Clin Oncol 25:A3529.

PUBLICATIONS

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APPENDICES

1. **Chen, X,** Weaver, J., Bove, BA, Vanderveer, LA, Weil, SC, Miron, A, Daly, MB, Godwin, AK. Allelic Imbalance in BRCA1 and BRCA2 Gene Expression Is Associated with an Increased Breast Cancer Risk. Hum Mol Genet, 17: 1336-1348, 2008.

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Allelic imbalance in *BRCA1* and *BRCA2* gene expression is associated with an increased breast cancer risk

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The contribution of *BRCA1* and *BRCA2* to familial and non-familial forms of breast cancer has been difficult to accurately estimate because of the myriad of potential genetic and epigenetic mechanisms that can ultimately influence their expression and involvement in cellular activities. As one of these potential mechanisms, we investigated whether allelic imbalance (AI) of *BRCA1* or *BRCA2* expression was associated with an increased risk of developing breast cancer. By developing a quantitative approach utilizing allele-specific real-time PCR, we first evaluated AI caused by nonsense-mediated mRNA decay in patients with frameshift mutations in *BRCA1* and *BRCA2*. We next measured AI for *BRCA1* and *BRCA2* in lymphocytes from three groups: familial breast cancer patients, non-familial breast cancer patients and age-matched cancer-free females. The AI ratios of *BRCA1*, but not *BRCA2*, in the lymphocytes from familial breast cancer patients were found to be significantly increased as compared to cancer-free women (*BRCA1*: 0.424 versus 0.211, $P = 0.00001$; *BRCA2*: 0.206 versus 0.172, $P = 0.38$). Similarly, the AI ratios were greater for *BRCA1* and *BRCA2* in the lymphocytes of non-familial breast cancer cases versus controls (*BRCA1*: 0.353, $P = 0.002$; *BRCA2*: 0.267, $P = 0.03$). Furthermore, the distribution of under-expressed alleles between cancer-free controls and familial cases was significantly different for both *BRCA1* and *BRCA2* gene expression ($P < 0.02$ and $P < 0.02$, respectively). In conclusion, we have found that AI affecting *BRCA1* and to a lesser extent *BRCA2* may contribute to both familial and non-familial forms of breast cancer.

INTRODUCTION

Breast cancer is the most common cancer affecting women, with a lifetime risk among females ~10% by the age of 80 years. In the USA, it has been reported that there will be approximately 180 510 new cases of breast cancer, and more than 40 910 breast cancer-related deaths in 2007 (1). Current estimates suggest that family history is associated with 10–20% of breast cancer (2,3). *BRCA1* (OMIM: 113705) and *BRCA2* (OMIM: 600185) are two of the most prominent breast cancer susceptibility genes and deleterious mutations in these two genes are estimated to account for about 15–30% of familial breast cancer (4–6).

Germline mutations affecting the coding region of *BRCA1* and *BRCA2* are thought to lead to expression of mutant proteins, which are either inactive or function as dominant negatives. However, these scenarios have not been supported by functional studies (7–9). In fact, *Brca1* and *Brca2* knockout mouse models have demonstrated that elimination of Brca1 or Brca2 proteins is sufficient for the development of mammary cancer (10,11). Previously, we have reported that mutant *BRCA1* mRNAs containing premature stop codons were eliminated or destabilized by nonsense-mediated mRNA decay (NMD) (12) and lead to a state of haploinsufficiency. As a result, the ratios between the expressions from the mutant alleles and the corresponding wild-type alleles were

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significantly decreased, resulting in what was referred to as allelic imbalance (AI). AI of *BRCA1* or *BRCA2* expression could decrease the level of both transcripts and proteins and thus contribute to increased susceptibility of developing breast cancer.

There is growing evidence to support this concept. Epigenetic studies have shown that loss of *BRCA1* expression due to promoter hypermethylation is associated with ~10% of sporadic cases of breast and ovarian cancer (13–18). However, screens to evaluate AI have not been applied in depth to study its potential role in the genesis of familial forms of these diseases. A previous study reported that 6 out of 13 human genes, including *BRCA1* and *p53*, were expressed with significant difference between the two alleles, and this difference was transmitted by Mendelian inheritance (19). Furthermore, Yan *et al.* (20) observed that decreased expression of one of the adenomatous polyposis coli tumor suppressor gene (*APC*) alleles was associated with the development of familial adenomatous polyposis. Their studies also found that even more modest decreases in the expression of one *APC* allele could contribute to attenuated forms of polyposis (20). Based on these findings, we hypothesize that a subset of non-*BRCA1/2* mutation carriers with a strong family history of breast cancer are at increased risk of developing this disease as a result of AI in *BRCA1* and *BRCA2* gene expression.

In the present study, we have developed a quantitative approach to measure the allele-specific expression of *BRCA1* and *BRCA2*. We compared *BRCA1/2* allelic variation in a cohort of *BRCA1/2* mutation-negative familial breast cancer patients, non-familial breast cancer patients and age-matched cancer-free volunteers. Since susceptibility to breast cancer is far from being fully understood, our study may help to further identify genetic factors which contribute to breast cancer susceptibility.

RESULTS

Development of a quantitative allelic imbalance assay

In order to determine if allele-specific real-time PCR is able to quantitatively measure the AI in *BRCA1* and *BRCA2* gene expression from the individual allele, RNAs were isolated from the blood lymphocytes of two individuals determined by genotype and sequence analysis to be homozygous for either *BRCA1*-c.4308T/T or *BRCA1*-c.4308C/C (Fig. 1A). This polymorphism was chosen since it is relatively common, based on NCBI dbSNP data. The samples were then reverse transcribed and the cDNAs were mixed at various ratios (8:1, 4:1, 2:1, 1:1, 1:2, 1:4 and 1:8) as described in the Materials and Methods section. *BRCA1*-c.4308T/T was detected by the VIC fluorescence signal and *BRCA1*-c.4308C/C was detected by the FAM fluorescence signal. As shown in Figure 1B, with decreasing cDNA ratios of c.4308T to c.4308C, the VIC curve (detecting the c.4308T allele) shifted to the right with the increasing value of $C_{T\text{-}c.4308T\ (VIC)}$, while the curve of FAM (detecting c.4308C allele) shifted to the left with the decreasing value of $C_{T\text{-}c.4308C\ (VIC)}$. At the same time, the value of ΔC_T ($C_{T\text{-}c.4308T\ (VIC)} - C_{T\text{-}c.4308C\ (FAM)}$) changed from the negative to the positive. By the regression analysis, a linear relationship between Log_2 ratio of cDNAs c.4308T to c.4308C and ΔC_T was identified: Log_2 (c.4308T/C) $= -0.0877 + 1.57897 * \Delta C_T$ ($P < 0.001$) (Fig. 1C). The Pearson correlation coefficient (r) between Log_2 (c.4308T/c.4308C) and ΔC_T was 0.9798. To establish a similar standard curve for *BRCA2* allelic expression, cDNAs from two individuals, who were either homozygous for *BRCA2*-c.3396A/A or *BRCA2*-c.3396G/G, were mixed at the following ratios: 8:1, 4:1, 2:1, 1:1, 1:2, 1:4 and 1:8 (c.3396A/A allele:c.3396G/G allele). *BRCA2*-c.3396A was detected by the VIC fluorescence signal and *BRCA2*-c.3396G was detected by the FAM fluorescence signal. As shown in Figure 1D, with decreasing ratios of c.3396A to c.3396G, the VIC curve (detecting c.3396A allele) shifted to the right while the FAM curve (detecting c.3396G allele) shifted to the left. After regression analysis, a linear relationship between Log_2 (c.3396A/c.3396G) and ΔC_T was identified: Log_2 (c.3396A/G) $= 0.11726 + 1.26458 * \Delta C_T$ ($P < 0.001$) (Fig. 1E). The Pearson correlation coefficient (r) between Log_2 (c.3396A/G) and ΔC_T was 0.9868.

Detection of allelic imbalance caused by nonsense-mediated mRNA decay

To examine whether the allele-specific real-time PCR assay is able to detect AI of *BRCA1* and *BRCA2* gene expression in cell lines, we evaluated RNAs isolated from lymphoblastoid cell lines (LCLs) which were derived from deleterious mutation carriers heterozygous for *BRCA1*-c.3671ins4 or *BRCA2*-c.796delT. These frame-shift mutations create the premature stop codons, which are predicted to activate the NMD pathway and thus lead to decreased levels of mRNAs from the mutant alleles (12). As shown in Figure 2A and B, the ratios of *BRCA1*-c.4308T to -c.4308C between wild type and *BRCA1*-c.3671ins4 heterozygous samples were 0.93 ± 0.04 and 2.07 ± 0.06, respectively ($P < 0.01$). By subcloning and sequencing the individual transcripts, we found that the under-expressed allele contained both the *BRCA1*-c.3671ins4 mutation and the *BRCA1*-c.4308C polymorphism (detected by the FAM signal) (data not shown). To further examine if the loss of *BRCA1*-c.3671ins4 was associated with NMD, we treated the *BRCA1*-c.3671ins4 LCLs with puromycin, a translational inhibitor, 14 h prior to RNA isolation. The ratio of *BRCA1*-c.4308T to -c.4308C in *BRCA1*-c.3671ins4 heterozygous cells decreased ~30%, in comparison to the non-treatment group (1.50 ± 0.05 versus 2.07 ± 0.06, $P < 0.01$) (Fig. 2B). Our data indicated that treatment with puromycin was able to partially recover the AI caused by NMD. Significant AI was also observed for the *BRCA2*-c.796delT mutant allele. The ratios of *BRCA2*-c.3396G to -c.3396A between wild-type and *BRCA2*-c.796delT heterozygous samples were 0.98 ± 0.06 and 6.59 ± 1.31, respectively ($P < 0.01$). After treating the *BRCA2*-c.796delT LCLs with puromycin, the ratio of *BRCA2*-c.3396G to -c.3396A in *BRCA2*-c.796delT heterozygous cells decreased ~31%, in comparison to the non-treatment group (4.90 ± 0.87 versus 6.25 ± 1.17) (Fig. 2C and D). Our results suggested that the loss of expression of *BRCA1* or *BRCA2* mutant alleles via NMD significantly contributed to the observed AI.

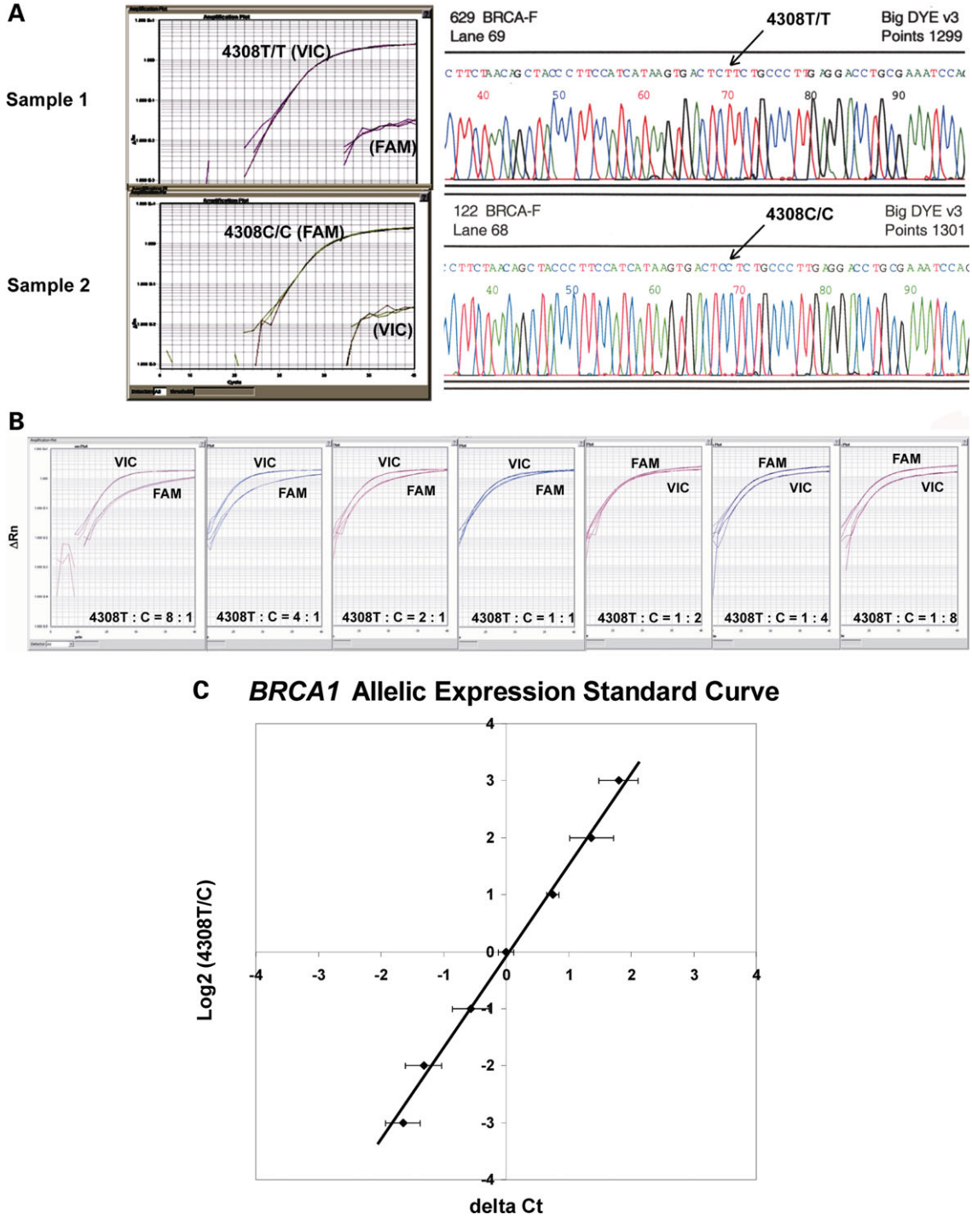

Figure 1. Standard curves for *BRCA1* and *BRCA2* allelic imbalance.
(**A**) Allele-specific real-time PCR amplification plot analyses of *BRCA1*-c.4308T (VIC) and -c.4308C (FAM) was performed in cDNAs generated by RT–PCR using RNAs from blood lymphocytes of two individuals homozygous for either the *BRCA1*-c.4308T/T or *BRCA1*-c.4308C/C. DNA sequencing chromatograms confirming the genotype are shown in the right panel. (**B**) Allele-specific real-time PCR amplification plot was analyzed in mixed cDNAs of *BRCA1*-c.4308T/T (detected by VIC) and *BRCA1*-c.4308C/C (detected by FAM) at the following ratios: 8:1, 4:1, 2:1, 1:1, 1:2, 1:4 and 1:8, respectively. (**C**) The standard curve for *BRCA1* allelic imbalance: Log_2 (c.4308T/C) = $-0.0877 + 1.57897 * \Delta C_T$. The Pearson correlation coefficient (*r*) between Log_2 (c.4308T/c.4308C) and ΔC_T was 0.9798 (Data expressed as Mean $\pm$ SD, *n*=3; the mean value of ΔC_T for c.4308T/C=1 has been adjusted to zero). (**D**) Allele-specific real-time PCR amplification plot was analyzed in mixed cDNAs of *BRCA2*-c.3396A/A (detected by VIC) and *BRCA2*-c.3396G/G (detected by FAM) at the following ratios: 8:1, 4:1, 2:1, 1:1, 1:2, 1:4 and 1:8, respectively. (**E**) The standard curve for *BRCA2* allelic imbalance: Log_2 (c.3396A/G) = $0.11726 + 1.26458 * \Delta C_T$. The Pearson correlation coefficient (*r*) between Log_2 (c.3396A/G) and ΔC_T was 0.9868 (Data expressed as Mean $\pm$ SD, *n* = 3; the mean value of ΔC_T for c.3396A/G = 1 has been adjusted to zero).

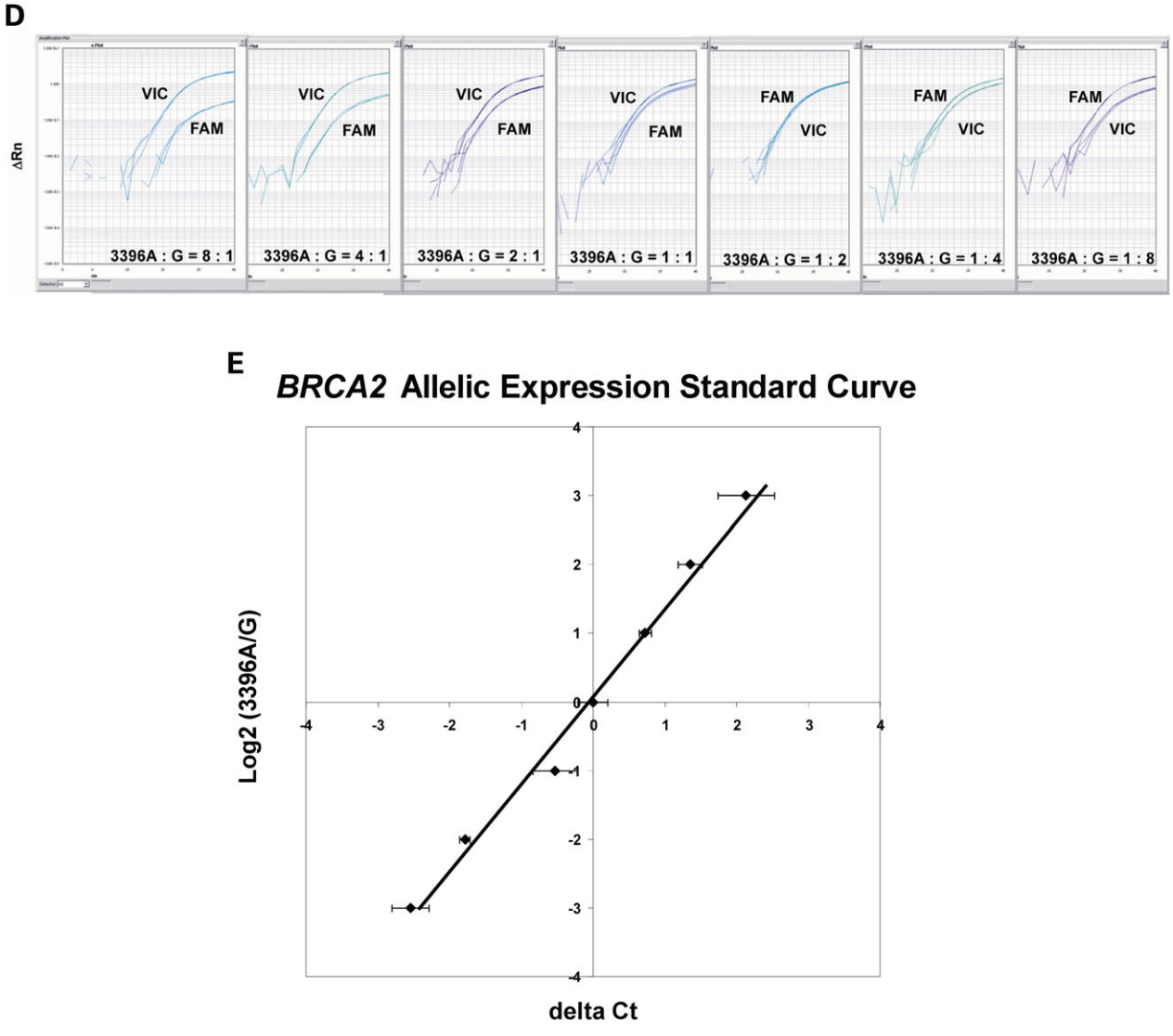

Figure 1. *continued*

BRCA1 and *BRCA2* allelic imbalance is associated with breast cancer risk

To evaluate AI of *BRCA1* and *BRCA2* gene expression, genotype analysis of the two common polymorphisms, *BRCA1*-c.4308T/C and *BRCA2*-c.3396A/G, was performed on DNA samples isolated from fresh-frozen peripheral blood lymphocytes from 85 unrelated *BRCA1/2* mutation-negative familial breast cancer carriers (median age at sample collection: 47), 112 non-familial breast cancer carriers (median age at sample collection: 52) and 102 age-matched cancer-free females (median age at sample collection: 51) (Table 1). From these analyses, 37 (43.5%), 48 (42.9%) and 41 (40.2%) of the samples evaluated were determined to be heterozygote for the *BRCA1*-c.4308T/C polymorphism for familial breast cancer patients, non-familial cancer patients and cancer-free controls, respectively (Table 1). Furthermore, 39 (45.9%), 44 (39.3%) and 36 (35.3%) of the samples above were found to be heterozygous for the *BRCA2*-c.3396A/G polymorphism (Table 1). Since our initial validation studies were preformed using immortalized LCLs, we first compared AI in RNA isolated from 20 fresh-frozen lymphocytes versus 20 established Epstein-Barr Virus (EBV)-lines. No significant differences were detected between these two sample sets [*BRCA1*: 0.424 ± 0.129 versus 0.409 ± 0.127 ($n = 11$); *BRCA2*: 0.212 ± 0.180 versus 0.225 ± 0.209 ($n = 10$)]. However, to limit any AI variation potentially introduced by EBV transformation, all subsequent AI assays were performed using RNAs isolated from peripheral blood lymphocytes. Next, RNA isolated from *BRCA1*-c.4308T/C ($n = 126$) and *BRCA2*-c.3396A/G ($n = 119$) heterozygotes, including single heterozygotes and double heterozygotes, were evaluated for integrity and quantity. Those samples demonstrating high quality and the necessary quantities were used in the AI assay, as described in the Materials and Methods section.

To evaluate the AI, we used the absolute values of Log_2 (*BRCA1*-c.4308T/C) or Log_2 (*BRCA2*-c.3396A/c.3396G).

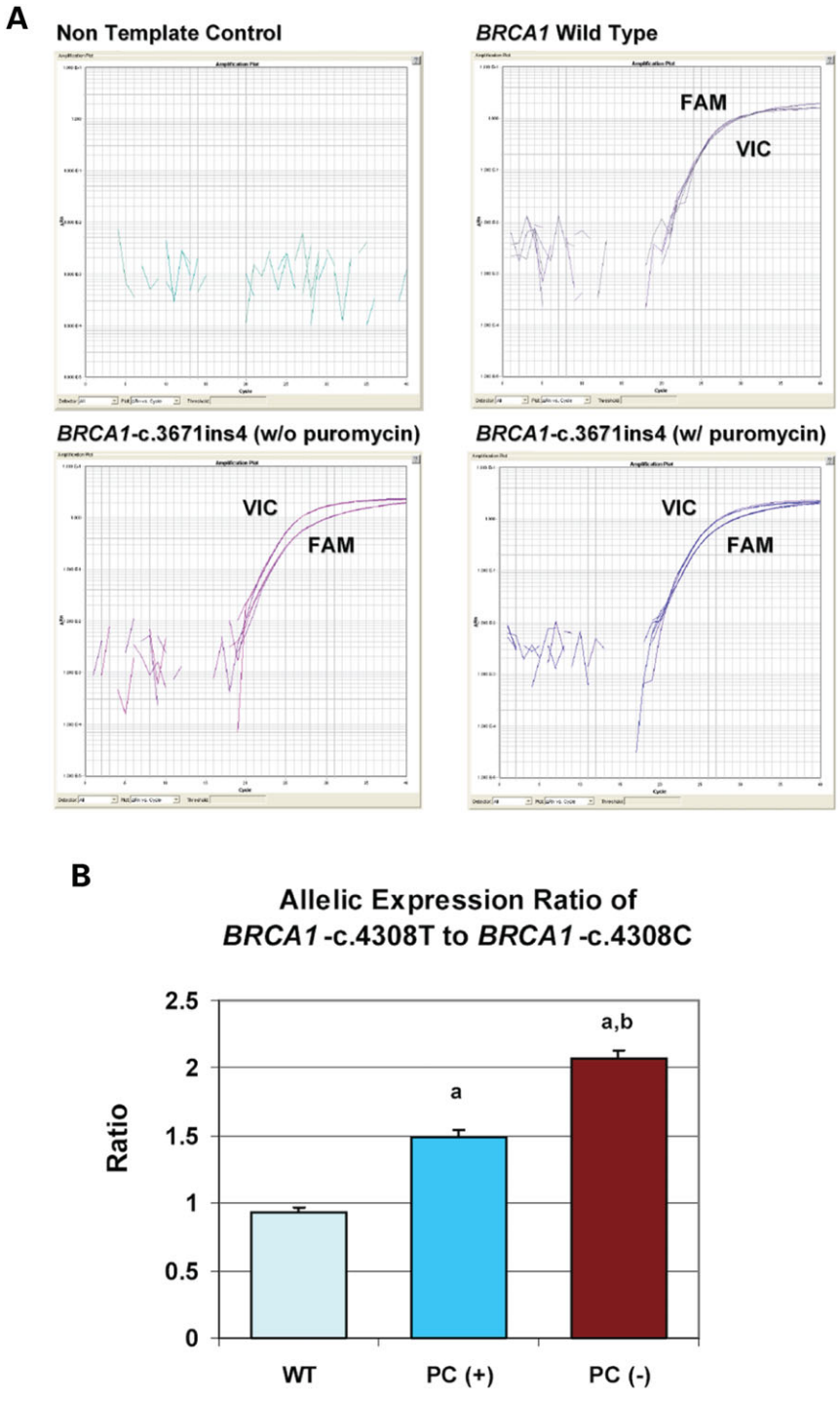

Figure 2. *BRCA1* and *BRCA2* allelic imbalance caused by NMD. (**A**) Allele-specific real-time PCR amplification plots of *BRCA1*-c.4308T (VIC) and -c.4308C (FAM) for non-template control, *BRCA1* wild-type lymphoblastoid cells (WT), *BRCA1* mutant (heterozygous *BRCA1*-c.3671ins4) lymphoblastoid cells without [PC (−)] or with [PC (+)] puromycin treatment. (**B**) Allelic expression ratios of *BRCA1*-c.4308T to *BRCA1*-c.4308C (a: versus WT; b: versus PC (+); *t*-test, $P < 0.05$). (**C**) Allele-specific real-time PCR amplification plots of *BRCA2*-c.3396A (VIC) and -c.3396G (FAM) for non-template control, *BRCA2* wild-type lymphoblastoid cells (WT), *BRCA2* mutant (heterozygous *BRCA2*-c.796delT) lymphoblastoid cells without [PC (−)] or with [PC (+)] puromycin treatment. (**D**) Allelic expression ratios of *BRCA2*-c.3396G to *BRCA2*-c.3396A (a: versus WT; b: versus PC (+); *t*-test, $P < 0.05$).

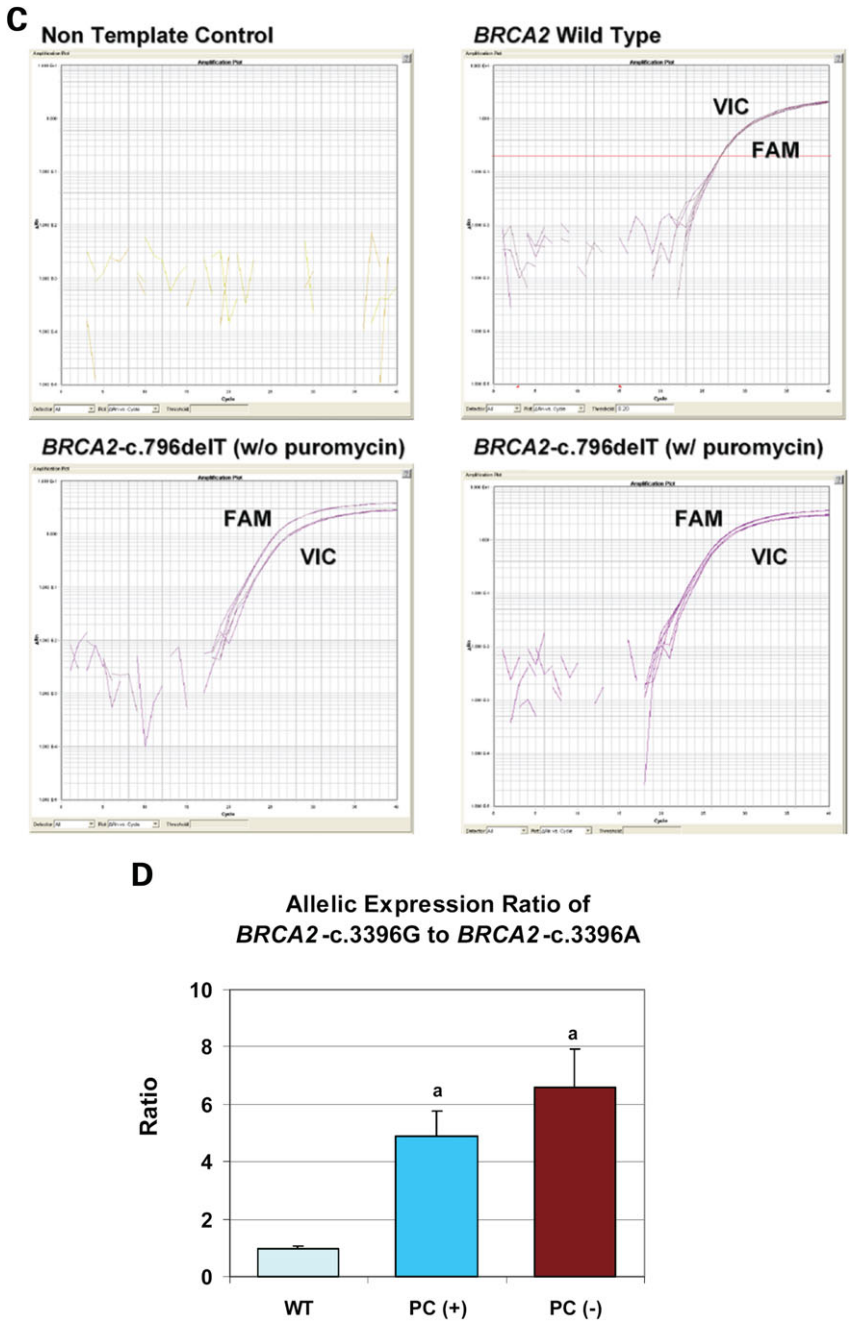

Figure 2. *continued*

The mean value of Log_2 (c.4308T/C) of *BRCA1* in the lymphocytes from familial breast cancer carriers was found to be ~104% higher than that that in the lymphocytes from cancer-free controls [0.424 ± 0.157 ($n = 32$) versus 0.211 ± 0.169 ($n = 40$), $P = 0.00001$; *t*-test] (Table 2 and Fig. 3A and B). Log_2 of *BRCA1*-c.4308T/C in the lymphocytes from non-familial breast cancer carriers was 73% higher than that in cancer-free controls [0.353 ± 0.209 ($n = 32$), $P = 0.002$ versus control] (Table 2 and Fig. 3A and C). In comparison, the mean value of Log_2 of *BRCA2*-c.3396A/G in the lymphocytes from familial breast cancer patients was moderately higher (10%) than that in cancer-free controls [0.206 ± 0.180 ($n = 37$) versus 0.172 ± 0.123 ($n = 31$), $P = 0.38$; *t*-test] (Table 2 and Fig. 4A and B). A similar result (38% higher) was observed for Log_2 (c.3396A/G) of *BRCA2* in the lymphocytes of non-familial breast cancer carriers [0.267 ± 0.171 ($n = 26$), $P = 0.03$ versus control] (Table 2 and Fig. 4A and C).

Interestingly, the distribution of under-expressed alleles of *BRCA1* and *BRCA2* was found to be significantly different between cancer-free control and familial breast carriers, but not between cancer-free control and non-familial breast carriers. As shown in Table 3 and Figure 3, under-expressed

Table 1. Characteristics of the study groups

Characters	Study groups		
	Familial	Non-familial	Cancer-free
Sample size	85	112	102
Age (median)			
At diagnosis	44	49	NA
At sample collection	47	52	51
Family history[a]			
2 or more	85	0	0
1	0	23	25
0	0	89	77
Genotypes			
BRCA1-c.4308T/C	37	48	41
BRCA2-c.3396A/G	39	44	36

[a]Number of first and/or second-degree relatives affected with either breast and/or ovarian cancer.

BRCA1-c.4308T (i.e. Log_2 [4308T/C] < 0) and *BRCA1*-c.4308C (i.e. Log_2 [4308T/C] > 0) alleles were found in ~53% (21 of 40) and ~47% (19 of 40) of cancer-free controls as compared to ~28% (9 of 32) and ~72% (23 of 32) of familial breast cancer carriers, respectively ($P < 0.02$). In addition, under-expressed *BRCA2*-c.3396A [i.e. Log_2 (3396A/G) < 0] and *BRCA2*-c.3396G [i.e. Log_2 (3396A/G) > 0] alleles were found in ~45% (14 of 31) and ~55% (17 of 31) of cancer-free controls as compared to ~70% (26 of 37) and ~30% (11 of 37) of familial breast cancer carriers ($P < 0.02$), respectively (Table 3 and Fig. 4).

Inheritance effects of AI in *BRCA1*

A previous study has indicated that AI for several tumor suppressor genes could be transmitted by Mendelian inheritance (19). To test if the AI observed in our study may be inherited, we identified three affected women (i.e. probands) reporting a significant family history of breast and/or ovarian cancer for which we had blood from at least one of their sisters. Furthermore, each sister had to be heterozygous for the *BRCA1*-c.4308T/C polymorphism. As shown in family A (Table 4), sister Sis-02 displayed a similar AI pattern as compared to the proband, while sister Sis-01 displayed no AI. In the two other families, both the affected probands and their corresponding sisters showed AI (Table 4). We further performed a haplotype analysis to determine whether the alleles showing AI were shared between siblings. As shown in Table 4, sisters with the same AI phenotype shared the same haplotype with their affected sister. Importantly, sister Sis-01 in family A did not share the same haplotype. Her blood sample displayed no AI (0.007 ± 0.147) for *BRCA1* gene expressions whereas the AI was detected in her unaffected and affected sisters (Sis-02 and Proband, 0.382 ± 0.176 and 0.375 ± 0.06, respectively) (Table 4). The allele frequencies of the microsatellite markers used for haplotype construction are listed in Supplementary Material, Table S1.

DISCUSSION

In this study, we developed a quantitative AI assay to examine the expression difference between the alleles of *BRCA1* and *BRCA2* (Fig. 1). By performing this AI assay with specific primers and probes that target common single nucleotide polymorphisms in *BRCA1* and in *BRCA2*, we were able to detect allelic imbalance associated with NMD in patients carrying frameshift mutations in *BRCA1* and *BRCA2* (Fig. 2). We next compared AI of *BRCA1* and *BRCA2* expression among three groups, familial breast cancer patients, non-familial breast cancer patients, and age-matched cancer-free females. AI ratios of *BRCA1* in familial breast cancer cases were significantly higher than those from cancer-free controls ($P = 0.00001$) (Table 2 and Fig. 3). Similar results were observed for AI ratios of *BRCA1* in the lymphocytes from non-familial breast cancer patients ($P=0.002$). AI ratios of *BRCA2* in familial or non-familial breast cancer cases were also higher than those from cancer-free controls ($P = 0.38$ or $P = 0.03$, respectively). However, the difference was not statistically significant in the ratios of mRNA expressed from the *BRCA2* alleles found in familial breast cancer cases when compared to cancer-free controls (Table 2 and Fig. 4). In addition, the distribution of under-expressed alleles between cancer-free controls and familial cases was significantly different for both *BRCA1* and *BRCA2* gene expression ($P < 0.02$ and $P < 0.02$, respectively) (Table 3). Furthermore, we have demonstrated that the AI patterns for *BRCA1* expression, albeit in a small number of families, can be transmitted by Mendelian inheritance (Table 4). Although these findings are consistent with a previous study (19), future evaluations will benefit from evaluating AI in large families for evidence of disease segregation.

Several methods have been developed to evaluate allele-specific expression. The first method combines primer extension and capillary electrophoresis (19,21). The second approach utilizes microarray technology to measure allele-specific mRNA expression (22). Compared to the AI assay presented here, the method of primer extension plus capillary electrophoresis is also accurate but relatively time-consuming and expensive. The microarray approach provides a high-throughput and a powerful platform for the simultaneous analysis of large numbers of genes to analyze allele-specific gene expression, but it has less power to define the AI. Like the majority of allelic expression methods (23), our AI assay also requires a transcribed heterozygous variant in the individuals to be evaluated. In the present study, we targeted two common polymorphisms, *BRCA1*-c.4308T>C and *BRCA2*-c.3396A>G in the general population. Therefore, a substantial number of subjects homozygous for the polymorphisms had to be excluded. To overcome this limitation of population selection based on genotypes, other primers and probes will need to be developed to target other common polymorphisms in *BRCA1* and/or *BRCA2*. In addition, our approach could easily be applied for studying AI in other cancer susceptibility genes, such as *p53*, *APC*, *PTEN,* etc.

In this study, we have demonstrated AI for both *BRCA1* and *BRCA2* in breast cancer populations. Interestingly, the increase of AI ratios in familial and non-familial breast cancer patients was more significant for *BRCA1* than *BRCA2*. Loss of *BRCA1* expression in breast cancer has been reported to be related to the pathogenesis of breast cancer (13–17). Loss of *BRCA2* expression in cancers, in

Table 2. Allelic imbalance in *BRCA1* and *BRCA2* expression

Genes	Population	Sample number	AI (Mean ± SD)[a]	*t*-test (*P*-value) versus cancer-free
BRCA1	Cancer-free	40	0.211 ± 0.169	
	Familial	32	0.424 ± 0.157	0.00001
	Non-familial	32	0.353 ± 0.209	0.002
BRCA2	Cancer-free	31	0.172 ± 0.123	
	Familial	37	0.206 ± 0.180	0.38
	Non-familial	26	0.267 ± 0.171	0.03

[a] To calculate the mean value of AI, all negative value of Log_2 (*BRCA1*-c.4308T/C) and Log_2 (*BRCA2*-c.3396A/c.3396G) in Figures 3 and 4 were changed to positive values.

contrast, is still controversial (24,25). These findings indicate that AI in *BRCA1* appears to be a more common event in breast cancer development than AI involving *BRCA2*. However, the mechanism(s) leading to the observed AI is for the most part unknown.

We have demonstrated that both *BRCA1* and *BRCA2* deleterious mutations can activate the NMD pathway and result in AI [Figure 2, and (12)]. However, all the familial breast cancer patients evaluated in the current study were determined to lack a mutation in *BRCA1* and *BRCA2* that would trigger NMD. Furthermore, we evaluated the *BRCA1* and *BRCA2* genes in the sporadic breast cancer patients and cancer-free controls demonstrating AI [i.e. allele expression ratio > 0.25 or < -0.25 (Figs 3 and 4)]. Again, no deleterious germline mutations were detected (data not shown). This is not entirely surprising given that germline mutations in *BRCA1* and *BRCA2* are rare in women affected with breast cancer without a strong family history of the disease (26–29).

Based on these observations, we conclude that NMD is not likely to be responsible for the observed AI in our case–control comparisons. Therefore, other mechanisms are likely to exist to account for the observed increased AI of *BRCA1* and *BRCA2* gene expression in female breast cancer patients. For example, the 5′ and 3′ non-coding regions of *BRCA1* and *BRCA2* are rarely evaluated through genetic testing, even though genetic alterations in these non-coding regions could be important in regulating *BRCA1* and *BRCA2* expression. For instance, genetic alterations within 5′ DNA or the putative promoter regions are able to disrupt the binding of transcription factors to DNA regulatory elements and hence lead to the loss of allelic gene expression. Several studies have shown that large genomic deletions involving the *BRCA1* promoter were associated with hereditary breast cancer (30–32). This concept is further supported by studies of Cowden syndrome (CS) showing that ~10% of CS-related *PTEN* mutations occur in the *PTEN* promoter and lead to a 50% reduction in PTEN expression (33,34). Also, allele-specific hypermethylation of the *BRCA1* promoter region and decreased *BRCA1* expression is associated with ~10% of sporadic breast cancer cases (18,30,35). Recent advances have identified a new pathway for gene regulation, i.e. via microRNAs (miRNAs) (36,37). These 21–22 nt RNA molecules are complementary to the 3′-UTR sequence of transcripts and mediate negative post-transcriptional regulation through RNA duplex formation (36,38). By performing *in silico* analyses in four *BRCA1* SNPs and two *BRCA2* SNPs (39), we have identified three rare *BRCA1* alleles (c.5628G, c.6273T, c.6924A) that could potentially create target sites for selected microRNAs (Supplementary Material, Table S2). Therefore, it is possible that altered mRNA targeting could contribute to AI of *BRCA1* gene expression in the absence of frameshift mutations. It will be important in future studies to determine the mechanisms that either disrupt transcription factors binding or alter miRNA binding, leading to constitutively decreased levels of *BRCA1* and *BRCA2* and an increased risk of developing breast cancer.

In summary, we have developed a quantitative approach to evaluate expression of *BRCA1* and *BRCA2* from individual alleles, and we have found that AI in *BRCA1* and to a lesser extent *BRCA2* is associated with increased breast cancer risk. Furthermore, we have demonstrated that the AI patterns for *BRCA1* expression could be transmitted by Mendelian inheritance. Since susceptibility to breast cancer is far from being fully understood, our study suggests that alternate mechanisms, other than deleterious coding mutations, may contribute to breast cancer.

MATERIALS AND METHODS

Databases

RefSeqs (GenBank Accession No: NM_007295.2 and NM_000059.1) were used for *BRCA1* and *BRCA2* mRNA numbering, respectively. The A of ATG translation initiation codon is defined as position +1.

Subjects and genotype analysis

Three populations were used in this study, (i) *BRCA1/2* mutation-negative women reporting a personal and family history of breast cancer, i.e. familial; (ii) female breast cancer patients without a significant family history of disease, i.e. non-familial; and (iii) age-matched cancer-free female controls (Table 1). All participants were Caucasian women with European-American ancestry and were from the Delaware Valley, including the greater Philadelphia Metropolitan area in Pennsylvania. For family studies, eligible subjects were women with a personal and family history of cancer (at least two first and/or second-degree relatives affected with either breast and/or ovarian cancer) and were ascertained from the Family Risk Assessment Program (FRAP) at the Fox Chase Cancer Center (FCCC). All relevant institutional

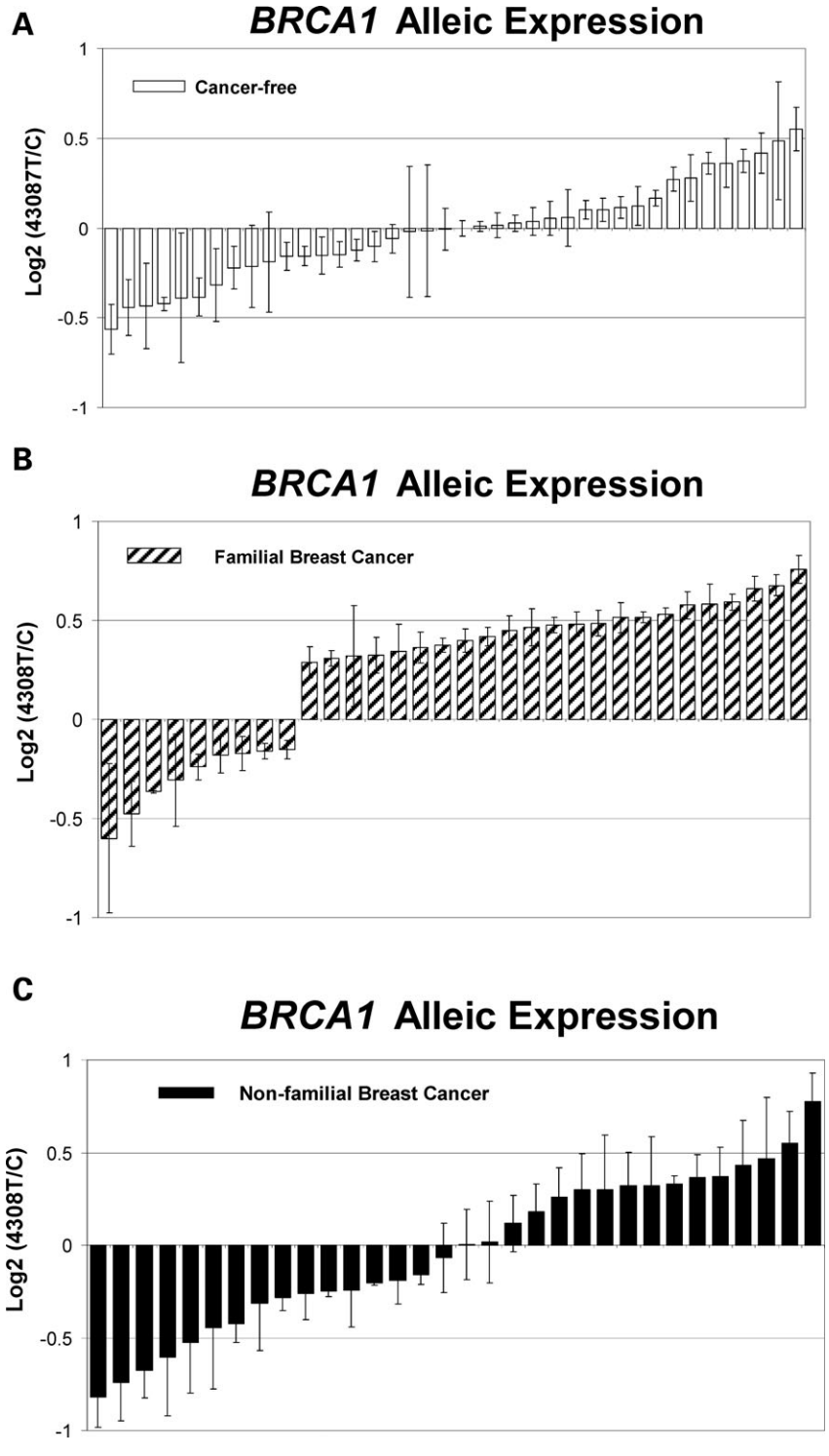

Figure 3. *BRCA1* allelic expression ratios in cancer-free controls, familial and non-familial breast cancer patients. The AI assays were performed using specific primer and probe sets targeting *BRCA1*-c.4308T/C alleles. Log_2 ratios of *BRCA1*-c.4308T allele to -c.4308C allele expression were presented in cancer-free controls (**A**), familial (**B**) and non-familial breast cancer patients (**C**). (Data expressed as Mean $\pm$ SD, $n = 3$; the mean value of allelic expression ratios of total normal samples has been adjusted to zero).

review boards approved the study protocol and written informed consent was obtained from all participants. Genotype analyses of the two common polymorphisms, *BRCA1*-c.4308T/C and *BRCA2*-c.3396A/G were carried out using ABI PRISM 7900HT Sequence Detection System and Assays-on-Demand SNP Genotyping products for fluorogenic polymerase chain reaction allelic discrimination (Applied Biosystems, Foster City, CA, USA).

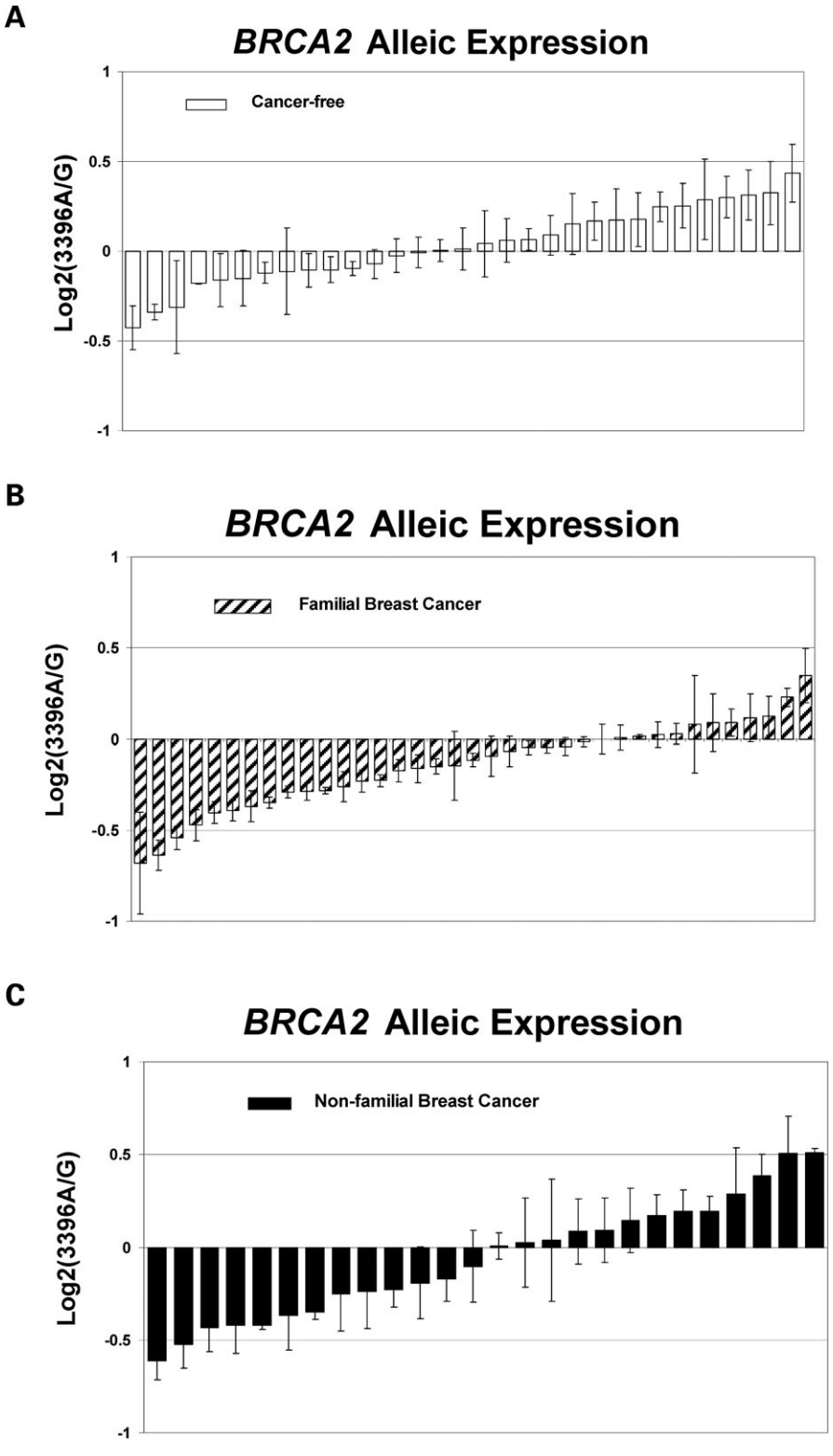

Figure 4. *BRCA2* allelic expression ratios in cancer-free controls, familial and non-familial breast cancer patients. The AI assays were performed using specific primer and probe sets targeting *BRCA2*-c.3396A/G. Log_2 ratios of *BRCA2*-c.3396A allele to -c.3396G allele expression were presented in cancer-free controls (**A**), familial (**B**) and non-familial cancer patients (**C**). (Data expressed as Mean $\pm$ SD, $n = 3$; the mean value of allelic expression ratios of total normal samples has been adjusted to zero).

Table 3. Distribution of under-expressed alleles of *BRCA1* and *BRCA2*

Genes	Group	Under-expressed alleles		OR (95% CI)	*P*-value[a]
BRCA1		c.4308T, Log_2 [4308T/C]<0	c.4308C, Log_2 [4308T/C]>0		
	Cancer-free Controls	21	19		
	Familial	9	23	2.82 (1.05, 7.60)	0.02
	Non-Familial	16	16	1.11 (0.44, 2.80)	0.18
BRCA2		c.3396A, Log_2 [3396A/G]<0	c3396G, Log_2 [3396A/G]>0		
	Cancer-free Controls	14	17		
	Familial	26	11	0.35	0.02
	Non-familial	13	13	0.82	0.20

[a]A χ^2 test was used to assess the 2 by 2 tables.

Table 4. Allelic expression and haplotype analysis of *BRCA1* in sisters from three breast cancer-prone families

Family	Members	Allelic expression [Log_2 (*BRCA1*-c.4308T/C)]	Haplotypes D17S855–D17S1322–D17S1325
Family A	Proband[a]	0.375 ± 0.060	145/155–121/121–193/193
	Sis-01	0.007 ± 0.147	145/153–121/121–195/193
	Sis-02	0.382 ± 0.176	145/155–121/121–193/193
Family B	Proband[b]	0.477 ± 0.070	145/151–121/124–193/193
	Sis-01	0.232 ± 0.214	145/151–121/124–193/193
Family C	Proband[b]	0.583 ± 0.243	145/153–121/127–189/189
	Sis-01	0.522 ± 0.156	145/153–121/127–189/189

[a]Ovarian cancer carrier.
[b]Breast cancer carrier.

Allelic imbalance assay

A 1.25 μl of the cDNA synthesized in the RT reaction was used in a real-time PCR reaction (25 μl total volume), performed with ABI PRISM 7900HT Sequence Detection System following methods recommended by the manufacturer. Optimal conditions were as follows: Step 1, 95°C for 10 min; Step 2, 92°C for 15 s, 60°C for 60 s with Optics; repeated for 40 cycles. The primer and probe sets used in real-time PCR reaction to detected *BRCA1*-c.4308T/C (rs1060915) and *BRCA2*-c.3396A/G (rs1801406) allelic expression were obtained from Applied Biosystem TaqMan® SNP Assay program (Assay ID: C.3178676 and C.7605673.1 for *BRCA1* and *BRCA2*, respectively). Sequence information for primers and probes is available upon request. Each 96-well PCR plate included negative controls, positive controls and unknown samples. Real-time PCR data were analyzed with ABI SDS 2.2.2 software. In order to produce the *BRCA1* allelic expression standard curve, cDNAs from the two samples with homozygous genotypes, *BRCA1*-c.4308T/T and *BRCA2*-c.4308C/C, were mixed as the following ratios: 8:1, 4:1, 2:1, 1:1, 1:2, 1:4 and 1:8 (c.4308T/T allele:c.4308C/T allele). For the same purpose, cDNAs from the two samples with homozygous genotypes, *BRCA2*-c.3396A/A and *BRCA2*-c.3396G/G, were mixed as the following ratios: 8:1, 4:1, 2:1, 1:1, 1:2, 1:4 and 1:8 (c.3396A/A allele:c.3396G/G allele).

The principles of quantitative real-time PCR provide the basis of this linear relation between Log_2 ratio and ΔC_T established in our approach to detect AI (40,41). Previous data have shown that AmpliTaq DNA polymerase cleaves the matched and well-hybridized probe and target sequences and produces a fluorescent signal (42). In contrast, mismatches between a probe and target are expected to reduce the efficiency of probe hybridization, and AmpliTaq DNA polymerase is more likely to displace a mismatched probe without cleaving it, which does not produce a fluorescent signal.

Theoretically, Allele 1 gene copy number (detected by FAM):

$$\mathrm{Log}_2[\mathrm{Allele}-1] = -\mathrm{A}_1 * \mathrm{C}_{\mathrm{T}1} + \mathrm{B}_1 \quad (1)$$

and Allele 2 gene copy number (detected by VIC):

$$\mathrm{Log}_2[\mathrm{Allele}-2] = -\mathrm{A}_2 * \mathrm{C}_{\mathrm{T}2} + \mathrm{B}_2 \quad (2)$$

If the fluorescence probes have the same efficiency to hybridize with matched target sequence, that is, $A_1 = A_2 = A$, therefore,

$$\mathrm{Log}_2[\mathrm{Allele}-1/2] = \mathrm{A} * (\mathrm{C}_{\mathrm{T}2} - \mathrm{C}_{\mathrm{T}1}) + (\mathrm{B}_1 - \mathrm{B}_2) \quad (3)$$

The function (3) was confirmed by two standard curves, Log_2 (c.4308T/C) = −0.0877 + 1.57897 * ΔC_T and Log_2 (c.3396A/G) = 0.11726 + 1.26458 * ΔC_T, set up by our experimental data (Fig. 1). Besides using function (3) to calculate the ratio of mRNA expression between the two alleles, function (1) and function (2) are able to be applied for examining the absolute value of each allele mRNA expression. However, the direct analysis of single allele expression is often complicated by the potential variations between individuals with different environmental or physiological background rather than genetic factors. Comparing the relative expression levels of two alleles of the same gene within the same biologic sample will help to minimize these variations.

Peripheral blood lymphocytes and LCLs

Lymphocytes were isolated from peripheral blood and stored at −150°C until needed. None of the blood samples from breast cancer patients were collected at the time of chemo- or radiation therapy. In addition, a subset of cyropreserved lymphocytes from *BRCA1* or *BRCA2* mutation carriers (e.g. *BRCA1*-c.3671ins4 and *BRCA2*-c.796delT) or disease-free individuals were infected with EBV to establish immortal LCLs. LCLs were maintained in RPMI (GIBCO BRL) media supplemented with 20% fetal calf serum and antibiotics at 37°C, 5% CO_2 atmospheric condition and 95% humidity. The immortalized LCLs from cancer-free individuals that had been tested negative for mutations in *BRCA1* and *BRCA2* served as wild-type controls. To prevent potential degradation of unstable transcripts by NMD a translation inhibitor, puromycin (Sigma, St Louis, MO, USA) was added to the LCL cells as described in a previous study (12).

Subcloning the PCR product and sequence analysis

PCR fragments containing a common polymorphism and deleterious mutation were subcloned directly into pCR®4-TOPO vector (Invitrogen, Carlsbad, CA, USA). PCR was then performed to identify bacterial colonies containing appropriate inserts. Plasmid DNA was purified using QIAfilter™ Plasmid Maxi Kit (Qiagen Inc., Valencia, CA, USA) and the insert was sequenced using either the universal M13-primers or the primers for PCR reactions.

RNA isolation and reverse transcription (RT)

Total cellular RNAs were isolated from blood lymphocyte pellets using TRIzol reagent according to the protocols provided by the manufacturer (Invitrogen Corp., Carlsbad, CA, USA). Purified RNAs were further processed to remove any contaminating DNA (DNA-free kit, Ambion, Inc., Houston, TX, USA). After quantification with Bioanalyzer-2100 system using RNA 6000 Nano LabChip kits (Agilent Technologies, Palo Alto, CA, USA), 2 μg of total RNA from each sample was used as a template to be reverse-transcribed (RT) in a 20 μl reaction [containing 5 μM random hexamers, 500 μM deoxynucleoside triphosphate mix, 1× RT (reverse transcriptase) buffer, 5 mM $MgCl_2$, 1.5 units of RNase inhibitor and 7.5 units of MuLV reverse transcriptase]. All reagents were purchased from Applied Biosystems (Branchburg, NJ, USA). The RT reaction conditions were 10 min at 25°C, 1 h at 42°C and 5 min at 94°C.

Haplotype analysis

Haplotypes were constructed for *BRCA1* using three polymorphic microsatellite repeat markers located within (D17S855 and D17S1322) or adjacent (D17S1325) to the *BRCA1* locus. The sequences of the primer pairs were obtained from the Genome Database (http://www.gdb.org) and PCR reaction was carried out as previously reported (43,44). PCR products with fluorescent dye (HEX) labeled primer were mixed with Hi-Di Formamide and a fluorescent labeled internal size marker. The mixture was subjected to electrophoreseis on an ABI 3100 Automated DNA Sequencer (Applied Biosystems, Foster City, CA, USA) and the data were analyzed by the GeneScan (Version 3.7) and GeneMapper (Version 4.0) software provided by the manufacturer.

Statistical analysis

Allele specific real-time PCR data were analyzed with ABI SDS software v2.2.2 (Applied Biosystems, Foster City, CA, USA). Statistical analysis was conducted using the SAS System (version 9) developed by the SAS Institute, Inc. (Cary, NC, USA). Student's *t*-test was employed for continuous data and results were presented as the mean ± SD. We compared the distribution of under-expressed alleles in *BRCA1* or *BRCA2* between cases and controls using χ^2 95% confidence intervals (CI) and the difference in distribution of under-expressed alleles was estimated as odds ratios (OR). A value of $P < 0.05$ is considered significant.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None of the authors has any conflict of interest.

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BRCA1-Associated Proteins: Novel Targets for Breast Cancer Radiation Therapy

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Abstract

Tumor cells, in general, are genomically unstable and have defects in DNA repair pathways, which subsequently hinder DNA damage responses. It has been proposed that therapeutic strategies specifically targeting DNA repair pathway proteins may lead to an increased therapeutic index in tumor cells versus normal cells. The BRCA1 pathways are known to play a critical role in DNA repair; thus, breast tumors with defects in proteins associated with the BRCA1 pathways are believed to be more sensitive to DNA damage-based therapies. BRCA1 can interact directly or indirectly with other tumor suppressors, DNA damage sensors, ubiquitin ligase partners, and signal transducers to form multi-subunit protein complexes. These protein complexes are involved in a broad range of biological processes including DNA repair, cell cycle control, ubiquitination, and chromatin remodeling. Growing evidence suggests that mutation and/or aberrant expression of one or more key members of the BRCA1-associated multi-protein complexes may result in loss of normal BRCA1 activity and disruption of the BRCA1 pathways. These BRCA1-associated proteins are potential modifiers of BRCA1 functions and, therefore, potential targets for sensitizing breast cancer cells to radiation therapy.

Keywords: *BRCA1, breast cancer, radiation resistance, DNA repair, cell cycle, ubiquitination, chromatin remodeling.*

Introduction

Breast cancer is the most common cancer affecting women, with an estimated lifetime risk of approximately 10% by 80 years of age. In the United States, it is estimated that approximately 182,450 new cases of female breast cancer will be diagnosed and greater than 40,000 breast cancer-related deaths will occur in 2008 (Jemal *et al.*, 2008). Approximately 13.2% of all American women (1 in 8) are expected to develop breast cancer sometime during their lifetime and 3.0% will subsequently die from the disease (Ries *et al.*, 2008). Despite advances in treatment and early detection, the breast cancer mortality rate among women in the United States decreased by only 2.2% per year between 1990 and 2002 (Jemal *et al.*, 2008).

Importantly, estimates from previous studies (Collaborative Group on Hormonal Factors in Breast Cancer, 2001; Margolin *et al.*, 2006) indicate that family history is associated with 15% to 20% of breast cancer cases in the United States. The *BRCA1* gene (OMIM: 113705) is one of the most intensively studied breast cancer susceptibility genes and has a profound role in breast cancer etiology owing to its involvement in several important cellular processes. Deleterious mutations in *BRCA1* are thought to account for approximately 10% to 20 % of hereditary breast cancers (Bove *et al.*, 2002; King *et al.*, 2003; Walsh *et al.*, 2006). Among its many biological functions, the BRCA1 protein is involved in DNA repair. Because DNA repair pathways and associated proteins are targeted by radiation therapy, there is considerable interest in the development of novel therapeutic strategies to sensitize breast cancer patients with mutations in *BRCA1* to radiation therapy. This article will provide an overview of BRCA1 and its associated

proteins with a particular emphasis on their role in DNA repair, as well as summarize current paradigms for breast cancer treatment with a focus on the development of new strategies to exploit the role of BRCA1 associated proteins and improve the efficacy of breast cancer radiation therapy.

Radiation Therapy for Breast Cancer

Current treatment paradigms for breast cancer are complex and reflect the considerable heterogeneity of the disease (NCCN breast cancer treatment guidelines 2008). Treatment options for noninvasive breast cancers range from observation alone, to breast-conserving lumpectomy with or without breast radiation, to total mastectomy depending on the tumor biology and individual patient risk. Radiation therapy is also used in the adjuvant setting and in combination with lumpectomy for locoregional treatment of early stage invasive breast cancers. Systemic treatment for advanced or metastatic breast cancer includes chemotherapy, endocrine therapy, and newer types of targeted therapeutic agents (e.g., targeted monoclonal antibodies and tyrosine kinase inhibitors). Radiation therapy has been a treatment modality for breast cancer patients for more than 100 years and, over the last 3 decades, has become a critical component of successful treatment strategies for breast cancer. An increasing role for radiation therapy developed in the early 1970s, when Fletcher documented that radiation therapy was instrumental in decreasing local recurrences (Fletcher, 1972). In particular, supraclavicular metastases were reduced from 20% to 25% to only 1.3% to 3% with the addition of ionizing radiation (IR). Radiation therapy has also been utilized to treat patients with tumors that have undergone total mastectomy resulting in a reduction in local recurrences by greater

than two-thirds (Fletcher, 1972). This early work led to an expanded role for radiation therapy in breast cancer.

The emergence of radiation therapy to the forefront of modern breast cancer treatment lies in its application in breast conservation therapy. Current NCCN treatment guidelines support the preferred use of breast conservation therapy (i.e., lumpectomy with or without breast radiation) as a breast treatment for the majority of women with early stage breast cancers (i.e., ductal carcinoma in situ, stage I and II breast cancers). Evidence suggests that the addition of radiation therapy may significantly reduce recurrence in this patient population. Landmark studies on the necessity of radiation therapy in breast conservation therapy came from Fisher and colleagues, as a part of a clinical trial conducted by the National Surgical Adjuvant Breast and Bowel Project (NSABP; Protocol B-06) that showed lumpectomy with radiation therapy had much lower recurrence rates than lumpectomy alone (10% versus 35%; $P < 0.001$) after 12 years of follow-up (Fisher *et al.*, 1995). This observation has been further validated by an extensive meta-analysis that supported improved local control with the addition of radiation therapy (Fisher *et al.*, 2002; Veronesi *et al.*, 2002). Recently, clinical research has examined the possible survival benefits of radiation therapy in breast cancer. The Early Breast Cancer Trialists' Collaborative Group (EBCTG) examined 78 trials involving more than 42,000 patients with breast cancer (Clarke *et al.*, 2006). In the analyses of trials directly comparing patients receiving radiation therapy versus those not receiving radiation therapy, a clear reduction in local recurrences occurred in the radiotherapy group, including patients undergoing mastectomy or breast conservation

therapy (Clarke *et al.*, 2006). Interestingly, there was also a notable improvement in survival among patients treated with radiotherapy. In fact, patients receiving radiotherapy for their breast cancer had a nearly 6% reduction in their 15-year breast cancer mortality risk and a 4% to 5% reduction in overall mortality (Clarke *et al.*, 2006). These findings support the contribution of radiotherapy to both the reduction of local recurrences and in 15-year overall mortality rates. Researchers have noted that breast cancer recurrences in the non-irradiated breast often occur within 3 years of initial diagnosis (Kurtz *et al.*, 1989). In comparison, local recurrences in irradiated breast tissue occur much later, with the risk increasing with time (7% risk at 5 years, 14% risk at 10 years, and 20% risk at 20 years) (Smith *et al.*, 2000). Thus, despite the benefits of radiation therapy in the treatment of breast cancer, patients continue to develop local recurrences in the targeted breast. The persistent recurrence of breast cancers following radiation therapy in multiple patient settings has prompted significant research efforts, particularly in understanding the etiology of radioresistant breast tumors and subsequent development of novel treatment paradigms to overcome this resistance.

Despite the benefits of radiation therapy in the treatment of breast cancer, patients continue to develop local recurrences in the targeted breast. Researchers have noted that breast cancer recurrences in the non-irradiated breast often occur within 3 years of initial diagnosis (Kurtz et al., 1989). In comparison, local recurrences in irradiated breast tissue occur much later, with the risk increasing with time (7% risk at 5 years, 14% risk at 10 years, and 20% risk at 20 years) (Smith et al., 2000). It is these recurrences that have spurred research into both breast cancer recurrences and the possibility of radio-resistant

breast tumors. One of the major radio-resistance mechanisms is related to a score of genes which are involved in the repair of DNA damage by radiation.

BRCA1 in Resistance to Breast Cancer Radiation Therapy

The clinical benefit of radiation therapy can be attributed to its mechanism of DNA damage and subsequent activation of apoptosis pathways. The damage caused by IR activates specific DNA damage cell cycle checkpoints, which leads to induction of various DNA repair pathways. The central component of these pathways is the ATM/CHEK2 kinase, which is activated upon DNA damage and subsequently phosphorylates multiple proteins, including BRCA1 (Canman *et al.*, 1998; Cortez *et al.*, 1999; Lee *et al.*, 2000). In response to DNA damage induced by IR, BRCA1 is phosphorylated at specific tyrosine residues by ATM (the gene mutated in ataxia telangiectasia), CHEK2 (the human homologue of yeast checkpoint protein kinase [hCds1]), or by the ATM-related kinase, ATR (Cortez *et al.*, 1999; Lee *et al.*, 2000; Tibbetts *et al.*, 1999). This phosphorylation, which occurs in a region containing clusters of serine-glutamine residues, has been shown to be functionally important using mouse models. In these studies, a mutated form of *BRCA1* lacking these phosphorylation sites failed to rescue radiation hypersensitivity when introduced into *BRCA1*-deficient cells (Cortez *et al.*, 1999). In addition, phosphorylation by ATM/CHEK2 following DNA damage is critical for the recruitment of BRCA1 to both DNA repair and chromatin remodeling protein complexes (Zhong *et al.*, 1999).

BRCA1 has been implicated in normal cellular processes, including DNA fidelity and damage repair, and has therefore been examined as having a possible role in the radioresistance of breast tumors. However, the specific role of BRCA1 in radioresistant breast cancer remains somewhat unclear. In vitro studies (Abbott *et al.*, 1999; Foray *et al.*, 1999; Mamon *et al.*, 2003; Ruffner *et al.*, 2001; Shen *et al.*, 1998) have demonstrated an increased sensitivity to IR when *BRCA1* is mutated in human breast cancer cell lines. However, clinical observations in breast cancer patients fail to reliably support these in vitro findings (Baeyens *et al.*, 2004; Garcia-Higuera *et al.*, 2001; Leong *et al.*, 2000). One study (Kirova *et al.*, 2005) found that *BRCA1* mutation carriers exhibited increased sensitivity to radiation therapy as assessed by the reduced rate of breast cancer recurrence following breast conserving treatment; however, Pierce and colleagues (Pierce *et al.*, 2000) noted no significant differences in local recurrences between *BRCA1* mutation carriers and patients with sporadic forms of breast cancer in a multicenter study. Two additional human studies (Baeyens *et al.*, 2004; Leong *et al.*, 2000) indicated that mutations in *BRCA1* may not account for clinical radiation hypersensitivity. These conflicting findings pose the question of whether *BRCA1* mutations will indeed increase the sensitivities of tumor cells to the radiation-based therapies. Therefore, the role of BRCA1 and its influence on tumor cell sensitivity to radiation in vitro and in vivo will require further investigation.

Role of BRCA1 and Associated Proteins in Breast Cancer Etiology

Since its cloning and characterization in the mid-1990s (Miki *et al.*, 1994), BRCA1 has been implicated in many cellular processes including DNA repair, cell-cycle-checkpoint

control, protein ubiquitination, and chromatin remodeling. Although mutations in *BRCA1* are known to contribute to the development of hereditary breast and ovarian cancers, *BRCA1* mutations in sporadic breast cancers, which account for approximately 90% of all breast cancers, are surprisingly rare (Futreal *et al.*, 1994). In this aspect, various studies have indicated that loss of *BRCA1* expression through epigenetic mechanisms may contribute about 10% of sporadic breast cancer (Esteller *et al.*, 2000; Rio *et al.*, 1999; Yang *et al.*, 2001). In addition, accumulating evidence suggests that dysfunction of other genes, coding for proteins in pathways complementary to BRCA1, may be important in the pathogenesis of a significant proportion of sporadic, non-hereditary cancers. This hypothesis is supported by several lines of evidence, including phenotypic analyses of breast and ovarian tumors, as well as mechanistic studies of BRCA1-associated pathways (Farmer *et al.*, 2005; Jazaeri *et al.*, 2002).

BRCA1-Associated Proteins: Functional Modifiers of BRCA1 Activity

Due to its clinical significance, the *BRCA1* gene is one of the most intensively studied breast cancer susceptibility genes. The *BRCA1* gene encodes for a 220 kDa nuclear phosphoprotein that has been suggested to play a role in maintaining genomic stability and to act as a tumor suppressor. The BRCA1 protein interacts directly or indirectly with other tumor suppressor proteins (e.g., p53 and BRCA2), DNA damage sensors (e.g., RAD51, RAD50, MRE11 and NBS1), signal transducers (e.g., p21 and cyclin B), and ubiquitination proteins (e.g., BARD1, BRCC36, and RAP80) to form multi-subunit protein complexes (**Figure 1**), such as the BRCA1-associated genome surveillance complex (BASC) and the BRCA1 and BRCA2 containing complex (BRCC). Importantly,

the proper formation of these multi-subunit protein complexes is critical in carrying out the multiple biological processes associated with BRCA1, including DNA repair, cell cycle control, chromatin remodeling, and ubiquitination.

The majority of BRCA1 functional studies have focused on its potential role in DNA damage responses. The implication that BRCA1 is a direct component of DNA damage response pathways comes from evidence of its interactions with BRCA2 and RAD51. The protein complex comprised of BRCA1, BRCA2, and RAD51 has been shown to activate DNA double-strand break (DSB) repair and to initiate homologous recombination, an observation which links the maintenance of genomic integrity to tumor suppression (Chen *et al.*, 1999). In addition, the BRCA1-associated MRE11-RAD50-NBS1 (MRN) complex has recently been demonstrated to activate CHEK2 downstream from ATM in response to replication-mediated DSBs (Takemura *et al.*, 2006). Disruption of any of these pathways may contribute to increased genomic instability and potentially sensitize cells to the effects of IR, specifically through the induction of cellular apoptosis.

The involvement of BRCA1 and its associated partners in normal DNA repair processes suggests that mutations in these tumor suppressor proteins would hinder DNA damage responses, predispose cells to additional accumulated mutations, and potentially contribute to subsequent malignant transformation. Importantly, compromised DNA repair mechanisms would also be expected to sensitize cells to the lethal effects of IR. Thus, while *BRCA1* mutations may play a profound role in breast cancer etiology, consequent disruption of normal DNA repair may actually be therapeutically exploited to

increase clinical radiation hypersensitivity in breast cancer patients who are *BRCA1* mutation carriers.

BRCA1 has also been shown to play a role in cell cycle control. For example, BRCA1 stimulates expression of the cyclin–dependent kinase (CDK) inhibitor, p21, and to inhibit cell-cycle progression into the S-phase (Somasundaram *et al.*, 1997). In addition, research has shown that BRCA1 is not only essential for activating the CHEK1 kinase that regulates G2/M arrest induced by DNA damage, but also controls the expression, phosphorylation, and cellular localization of Cdc25C and Cdc2/cyclin B kinases (Yarden *et al.*, 2002). Therefore, BRCA1 appears to be involved in regulating the onset of mitosis. Furthermore, a mouse study demonstrated that *BRCA1* knockout mice, generated by removal of exon 11, have a defective G_2/M cell cycle checkpoint and extensive chromosomal abnormalities (Xu *et al.*, 1999). It is also reported that elimination of one *Tp53* allele (*BRCA1 exon11*-/-;*Tp53*+/-) rescued the embryonic lethality caused by the deletion of *BRCA1* exon 11 and restored normal mammary gland development (Xu *et al.*, 2001). However, most female mice homozygous for the *Brca1* exon 11 deletion and heterozygous for loss of the *Tp53* gene developed mammary tumors within 6 to 12 months. Importantly, the resulting tumors lose the remaining *Tp53* allele (Xu *et al.*, 2001). These findings indicated that the genetic interactions between Brca1 and p53 are associated with breast carcinogenesis.

BRCA1 and its associated protein have also been found to be involved in the process of chromatin remodeling. Wang and colleagues (Wang *et al.*, 2000) used

immunoprecipitation and mass spectrometry to identify a large multi-subunit protein complex referred to as BASC (BRCA1-associated genome surveillance complex), which is comprised of ATM, BLM, MSH2, MSH6, MLH1, the RAD50-MRE11-NBS1 complex, and the RFC1-RFC2-RFC4 complex. Confocal microscopy demonstrated that BRCA1, BLM, and the RAD50-MRE11-NBS1 complex co-localized to large nuclear foci, and BASC has subsequently been shown to be involved in chromatin remodeling at sites of double-strand DNA breaks (Wang *et al.*, 2000). In addition, BRCA1 directly interacts with the brahma-related gene 1 (BRG1) subunit of SW1/SNF-associated complex which has been demonstrated to be involved in chromatin-remodeling (Bochar *et al.*, 2000). This finding links chromatin remodeling processes to breast cancer. Furthermore, the BRCT domain of BRCA1 has been reported to be associated with the histone deacetylases, HDAC1 and HDAC2 (Yarden and Brody, 1999). Collectively, these findings may help explain the involvement of BRCA1 in multiple, seemingly unrelated processes such as transcription and DNA repair.

BRCA1 also interacts with a number of proteins and displays significant ubiquitin ligase activities. Importantly, deleterious mutations affecting the BRCA1 RING-finger domain, found in clinical specimens, abolish the ubiquitin ligase activity of BRCA1 (Ruffner *et al.*, 2001; Wu *et al.*, 1996). These findings support a relationship between the ligase activity of BRCA1 and the predisposition to breast cancer. Using a combination of affinity purification of anti-FLAG and mass spectrometric sequencing, a multiprotein protein complex, termed BRCC (BRCA1/2 Containing Complex), which contains seven polypeptides including BRCA1, BRCA2, BARD1 and RAD51, has been identified

(Dong *et al.*, 2003). BRCC is an E3 ubiquitin ligase complex exhibiting activities in the E2-dependent ubiquitination of the tumor suppressor p53 (Dong *et al.*, 2003). In this multiprotein complex, one protein, referred to as BRCC36, has been found to be directly interacted with BRCA1. Cancer-causing truncations of BRCA1 have been found to abrogate the association of BRCC36 with BRCC (Dong *et al.*, 2003). We have also demonstrated that depletion of BRCC36 resulted in increased sensitivity in breast cancer cells to ionizing radiation (IR) and disruption of IR-induced BRCA1 phosphorylation and nuclear foci formation (Chen *et al.*, 2006). Previous study has shown that a recombinant four-subunit BRCC complex containing BRCA1-BARD1-BRCC45-BRCC36 revealed an enhanced E3 ubiquitin ligase activity compared to that of BRCA1-BARD1 heterodimer (Dong *et al.*, 2003). Furthermore, BRCC36 has recently been reported to also be present in a novel BRCA1-associated complex, BRCA1-BARD1-RAP80-Abraxas-BRCC36, which plays a role in recognizing DNA damage site (Wang *et al.*, 2007). These findings may suggest that the role of BRCC36 in DNA damage response could be dynamic and mediated by other protein partners (e.g., BRCC45, BRCC120, RAP80 or Abraxas) in the same complexes (**Figure 2**). In addition, BRCA1 has also been reported to interact with the RNA Pol II holoenzyme (Scully *et al.*, 1997). Two recent reports have suggested that BRCA1and BARD1 may be involved in the degradation of RNA polymerase II complex and siRNA-mediated knockdown of BRCA1 and BARD1 results in stabilization of RNAP II in the cells following UV exposure (Kleiman *et al.*, 2005; Starita *et al.*, 2005). These studies reported that BRCA1/BARD1 appears to initiate the degradation of stalled RNAP II and thus disrupts the coupled transcription by inhibiting RNA processing machinery in cells exposed to DNA damage. At present, the known substrates that are

polyubiquitinated by the BRCA1-BARD1 ubiquitin ligase are very limited and include RNA polymerase II, nucleophosmin/B23, and p53 (Dong *et al.*, 2003; Kleiman *et al.*, 2005; Sato *et al.*, 2004; Starita *et al.*, 2005).

BRCA1-associated Proteins as Potential Targets of Breast Cancer Therapies

In the last several decades, efforts have been made toward understanding the mechanism of response to both cytotoxic chemotherapy and radiation therapy in the treatment of breast cancer. Because tumor cells are typically genomically unstable with dysfunctional DNA damage responses, it has been proposed that targeting DNA repair pathways may lead to an increased therapeutic index in tumor cells versus normal cells. The involvement of BRCA1 and its associated partners in normal DNA repair processes suggests that mutations in these tumor suppressor proteins would hinder DNA damage responses, predispose cells to additional accumulated mutations, and potentially contribute to subsequent malignant transformation. Importantly, compromised DNA repair mechanisms would also be expected to sensitize cells to the lethal effects of IR. Thus, while *BRCA1* mutations may play a profound role in breast cancer etiology, consequent disruption of normal DNA repair may actually be therapeutically exploited to increase clinical radiation hypersensitivity in breast cancer patients who are *BRCA1* mutation carriers.

This speculation is supported by the recent development of the inhibitors of poly (ADP-ribose)-polymerase-1 (PARP). The PARP enzyme is involved in base excision repair which is critical pathway in the repair of DNA single-strand breaks (Ratnam and Low,

2007; Schreiber *et al.*, 2002). Farmer and colleagues have shown that defects in BRCA1 or BRCA2 profoundly sensitize cells to the inhibition of PARP enzymatic activity, resulting in chromosomal instability, cell cycle arrest, and subsequent apoptosis (Farmer *et al.*, 2005). PARP inhibitors are currently in clinical trials of patients with breast cancer or other malignancies who are *BRCA1* or *BRCA2* mutation carriers. Two phase I studies have shown that AZD2281 (AstraZeneca, UK), a potent orally active PARP inhibitor, is well tolerated and leads to significant PARP inhibition in patients carrying *BRCA1* and *BRCA2* mutations with breast or ovarian cancer (Fong *et al.*, 2008; Yap *et al.*, 2007). Importantly, clinical responses have been observed in all cohorts evaluated thus far, and future phase II studies are planned (Fong *et al.*, 2008; Yap *et al.*, 2007). Findings from these recent studies further suggest that the design of novel therapies, which inhibit components of particular DNA repair pathways, may provide effective and more tolerable therapeutic options for breast cancer patients with BRCA1 defects.

In vitro studies have demonstrated that breast cancer cells expressing mutated *BRCA1* have increased sensitivity to IR (Kennedy *et al.*, 2004; Powell, 2005). Notably, mutations in *BRCA1* itself may not be the only reason for loss of the encoded protein's activity. There is growing evidence that disruption of the BRCA1-associated multi-protein complexes, either through mutations or the aberrant expression of a key member(s) of these complexes, may result in loss of normal BRCA1 activity (Chen *et al.*, 2006; McCarthy *et al.*, 2003; Wang *et al.*, 2007; Wu *et al.*, 2007). In our own studies, we have tested the hypothesis that dysregulated expression (e.g., gain or loss) of protein(s) in BRCA1-associated pathways leads to a BRCA1 "null-like" phenotype and subsequent

DNA damage hypersensitivity in breast cancer cells (Chen *et al.*, 2006). As shown in **Figure 3**, BRCA1 and p53 are phosphorylated by the ATM kinase following IR. Depletion of the BRCA1-associated protein, BRCC36, prevents the phosphorylation of BRCA1 and disrupts BRCA1 nuclear foci formation following IR, an event that is associated with the induction of DNA repair. The proposed model illustrates that disruption of BRCA1 activation through depletion of BRCC36 may create an imbalance between the DNA repair and cell survival pathways and the apoptosis/cell death pathways following IR exposure. As a result, abrogation of BRCC36 sensitizes breast cancer cells to IR-induced apoptosis (Chen *et al.*, 2006).

This proposed mechanism is also supported by a number of studies that have demonstrated the impact of cellular resistance to IR upon manipulation of BRCA1-associated proteins, such as RAD51, MRE11, and NBS1 (**Table 1**) (Billecke *et al.*, 2002; Boulton *et al.*, 2004; Chinnaiyan *et al.*, 2005; Digweed *et al.*, 2002; Garcia-Higuera *et al.*, 2001; Houghtaling *et al.*, 2005; Kim *et al.*, 2007; Lio *et al.*, 2004; Liu *et al.*, 2007; Nakanishi *et al.*, 2002; Russell *et al.*, 2003; Sobhian *et al.*, 2007; Wang *et al.*, 2007; Yan *et al.*, 2008). In addition, because multiple genetic hits are necessary for tumorigenesis, individuals that carry defects in DNA damage repair/response genes are particularly cancer prone, due to the genetic instability and hypermutability of their cells (Deng, 2006; Jasin, 2002). Therefore, these BRCA1-associated proteins are likely to be involved in tumorigenesis and are potential therapeutic targets.

Summary

Since BRCA1 was cloned a decade ago, significant progress has been made in defining its biochemical and biological functions, as well as its role in breast and ovarian cancers. BRCA1 has been implicated in many cellular processes, including DNA repair, and protein ubiquitination. Because of the important role of BRCA1 in DNA repair, breast tumors with defective BRCA1 are believed to be more sensitive to DNA damage-based therapies. Nevertheless, defects in BRCA1 itself may not be the only reason for the loss of its activity nor the increased sensitivity of tumor cells to DNA damage-based agents. A number of studies have demonstrated that manipulation of BRCA1-associated proteins, such as RAD51, MRE11, and NBS1, can impact cellular sensitivity to IR. BRCA1-associated proteins may, therefore, be considered as potential targets for breast cancer therapies. Despite a potentially significant role for BRCA1-associated protein complexes in modifying the activities of BRCA1, the total number of complexes and the identity and function of component proteins has yet to be fully elucidated. Thus, much of the scientific effort related to BRCA1 is currently directed at defining the biochemical functions of BRCA1 in association with these protein complexes.

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Table 1. Radiation Sensitivity Studies Related to BRCA1-associated Proteins

Protein name	BRCA1 pathway affected	*BRCA1* gene manipulation approach	Increased resistance to IR	Increased sensitivity to IR
FANCD2	DNA repair	Defected		Garcia-Higuera et al, 2001 Houghtaling et al, 2005
NBS1	DNA repair	Defected	Nakanishi et al, 2002	
MRE11	DNA repair	Disrupted		Digweed et al, 2002
RAD51	DNA repair	Deficiency Blocking Overexpression	 Vispe et al, 1998	Lio et al, 2004 Russell et al, 2003
HDAC	Chromatin remodeling	Blocking		Chinnaivan et al, 2005
RB	Cell cycle checkpoint control	Decreasing Defected		Carlson et al, 2000 Billecke et al, 2002
BARD1	Ubiquitination	Depleted		Boulton et al, 2004
BRCC36	Ubiquitination	Depleted		Dong et al., 2003 Chen et al, 2006
RAP80	Ubiquitination	Depleted		Sobhian, et al, 2007 Yan et al, 2008
CCDC98/Abraxas	Ubiquitination	Deplete		Wang et al, 2007 Kim et al, 2007 Liu et al, 2007

IR, ionizing radiation.

Figure Legends

Figure 1. BRCA1-associated protein network. BRCA1 interacts with a number of proteins to form multi-subunit protein complexes, which are involved in DNA repair, cell cycle checkpoint control, protein ubiquitination, and chromatin remodeling.

Figure 2. BRCC36 in different BRCA1-associated protein complexes (BRCC or BRCA1 A Complex, respectively). Previous study has shown that BRCC36 potentiates the E3 ubiquitin ligase activity of BRCA1-BARD1 heterodimer. Recently, BRCC36 has been reported to also be present in a novel BRCA1-associated complex, BRCA1-BARD1-RAP80-Abraxas-BRCC36, which plays a role in recognizing DSB site.

Figure 3. A proposed model illustrating the role of BRCC36 in BRCA1-associated DNA repair pathway in response to ionizing radiation (IR). BRCA1 and p53 are phosphorylated by the ATM kinase following IR. The BRCA1 and p53 proteins are involved in DNA repair and apoptosis pathways, respectively. Depletion of the BRCA1-associated protein, BRCC36, prevents the phosphorylation of BRCA1 and disrupts BRCA1 nuclear foci formation following IR, an event that is associated with the induction of DNA repair. The proposed model illustrates that disruption of BRCA1 activation through depletion of BRCC36 may create an imbalance between the DNA repair and cell survival pathways and the apoptosis/cell death pathways following IR exposure. As a result, abrogation of BRCC36 sensitizes breast cancer cells to IR-induced apoptosis.

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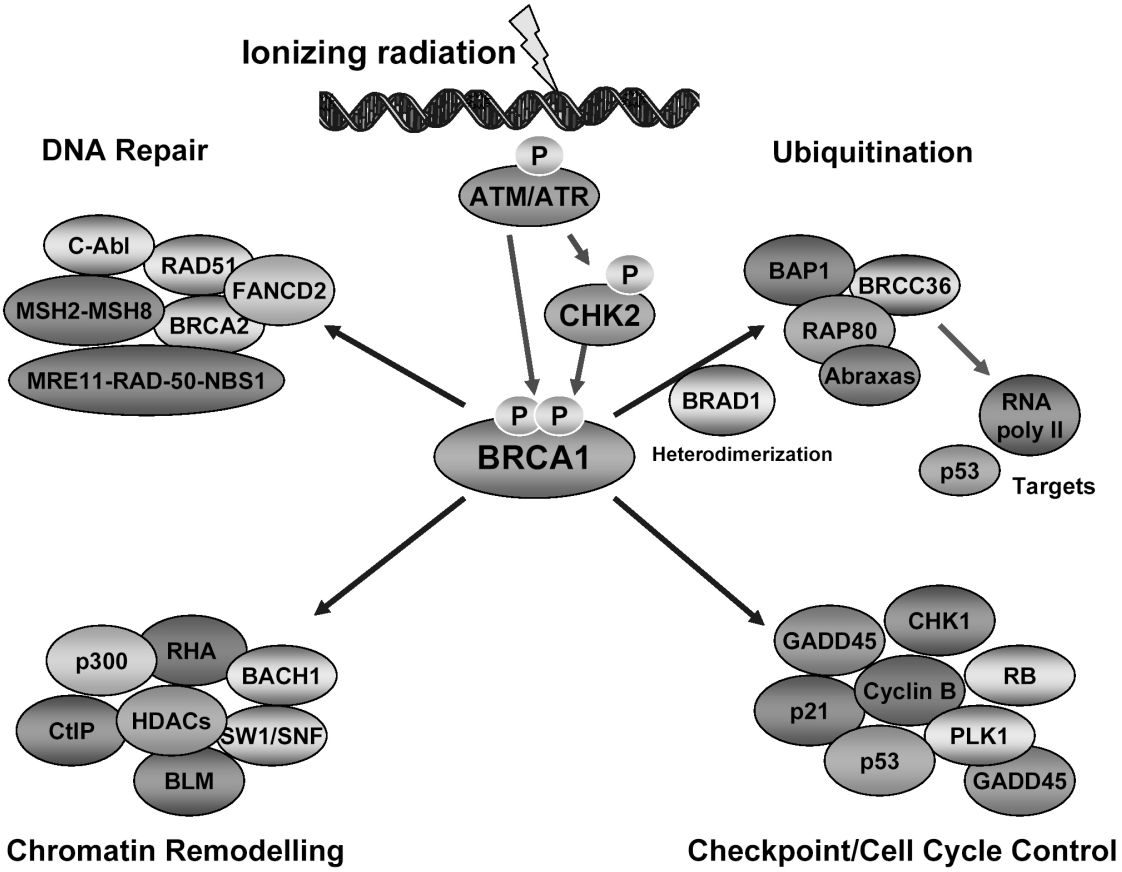
Figure 1


Ionizing radiation
DNA Repair
Ubiquitination
P
ATM/ATR
C-Abl
RAD51
FANCD2
MSH2-MSH8
BRCA2
MRE11-RAD-50-NBS1
P
CHK2
BAP1
BRCC36
RAP80
Abraxas
RNA poly II
p53
Targets
P P
BRCA1
BRAD1
Heterodimerization
p300
RHA
BACH1
CtIP
HDACs
SW1/SNF
BLM
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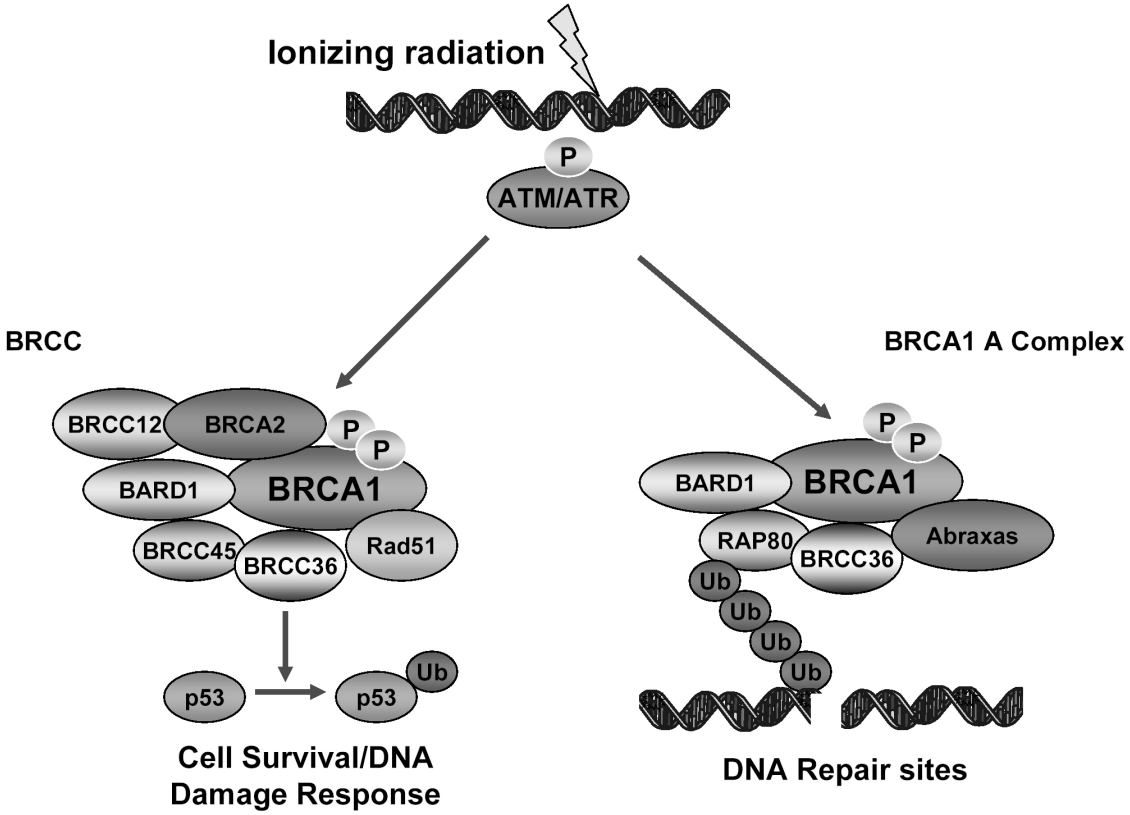
p53
GADD45
Chromatin Remodelling
Checkpoint/Cell Cycle Control

Figure 2


Ionizing radiation
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ATM/ATR
BRCC
BRCA1 A Complex
BRCC12
BRCA2
P
P
BARD1
BRCA1
BRCC45
BRCC36
Rad51
p53
p53
Ub
Cell Survival/DNA
Damage Response
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Abraxas
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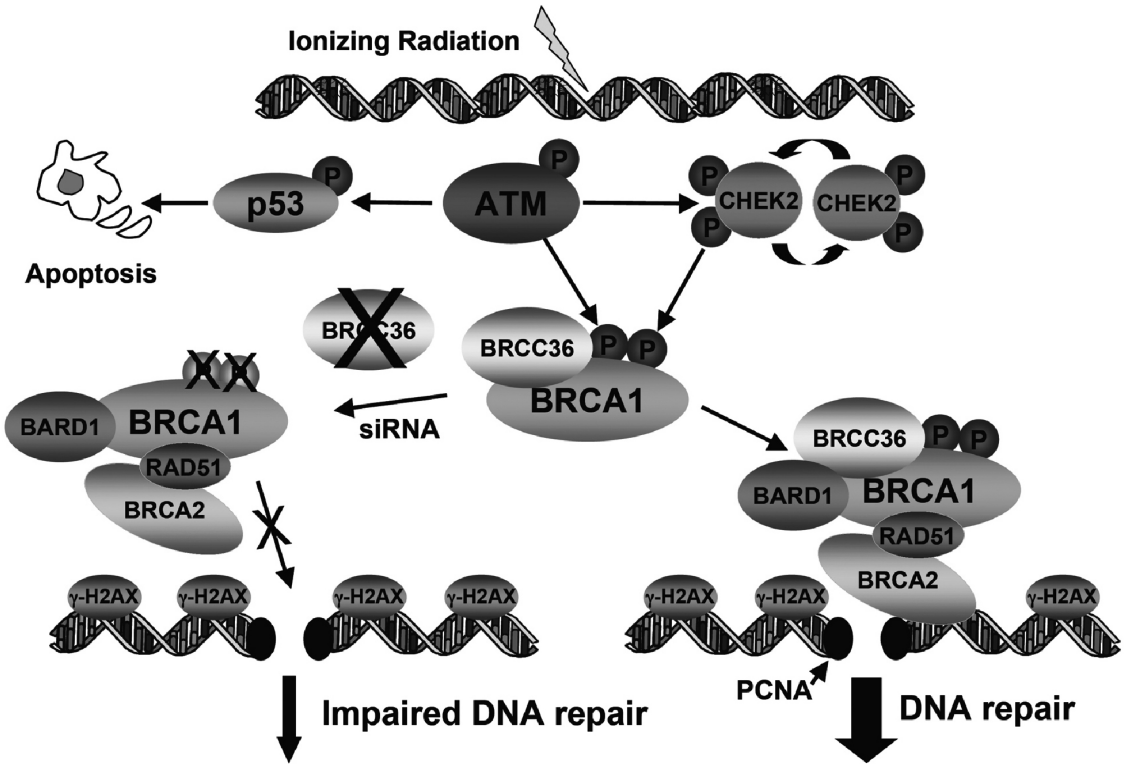
DNA Repair sites

Figure 3.


Ionizing Radiation
p53
ATM
CHEK2
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Apoptosis
BRCC36
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siRNA
BARD1
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RAD51
BRCA2
BRCC36
BARD1
BRCA1
RAD51
BRCA2
γ-H2AX
PCNA
Impaired DNA repair
DNA repair

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